Epstein-Barr Virus genome loss from Endemic Burkitt Lymphoma and its effect on cell phenotype

by

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Abstract

Epstein-Barr virus (EBV), a B cell growth-transforming human herpesvirus, is linked to several human malignancies, in particular endemic Burkitt lymphoma (eBL). Though always present in this tumour, EBV's role remains unclear since, in most cases, viral gene expression is restricted to the viral genome maintenance protein, EBNA1 and the non-coding EBERs, BARTs and BART-derived microRNAs (Latency I infection). This study first asked whether EBV was required for continued BL growth *in vitro* by screening a panel of Latency I BL cell lines for EBV-loss clones. Such clones were isolated from 5/12 BL lines tested. In each case these cells proved to be more sensitive to apoptosis than their EBV-positive counterparts, an effect which could be reversed by reinfection with a recombinant EBV. Cellular gene expression profiling of EBV-positive and EBV-loss clones on four BL backgrounds revealed transcriptional differences but none that were common to all four tumours. To examine the responsible viral function, a doxycycline-regulated vector was used to express EBNA1 and EBERs at physiologic and supra-physiologic levels in EBV-loss cells on two BL backgrounds. Contrary to previous reports, neither EBNA1 nor EBERs conferred apoptosis resistance, a result which implicates the BARTs or BART-derived microRNAs as novel anti-apoptotic effectors.

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List of Contents

List of figures

List of Tables

Abbreviations

αTG	alpha thioglycerol
aa	amino acid
ACV	acycloguanosine/acyclovir
AIDS	acquired immunodeficiency disease
AMV	avian myeloblastosis virus
APS	ammonium persulphate
ATP	adenosine triphosphate
B2m	beta 2 microglobulin
BARF0	*Bam*HI-A rightward frame 0
BART	*Bam*HI-A rightward transcript
BCR	B cell receptor
BCS	bathocupronine disulfonic acid
bHLH	basic helix loop helix
BL	Burkitt Lymphoma
BSAP	B cell lineage specific activator protein
C-terminal	carboxyl-terminal
CBP	Cp binding factor
Cdk	cyclin-dependent kinase
cDNA	complementary deoxyribonucleic acid
C_H	constant heavy
C_L	constant light
cRNA	complementary ribonucleic acid
CTAR	C-terminal activating region
CTD	C-terminal domain
CTL	cytotoxic T lymphocytes
DABCO	1,4-Diazobicyclo-(2,2,2)-octane
DD	death domain

DED	death effector domain
DEPC	diethylpyrocarbonate
D_H	diversity heavy
DISC	death-induced signalling complex
DLBCL	diffuse large B cell lymphoma
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
DS	dyad symmetry
DTT	dithiothreithol
EBER	Epstein-Barr virus-encoded small RNA
EBNA	Epstein-Barr nuclear antigen
eBL	Endemic Burkitt Lymphoma
EBV	Epstein-Barr virus
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
FACS	fluorescence activated cell sorting
FADD	Fas-associated death domain protein
FC	fold change
FCS	foetal calf serum
FISH	fluorescent in situ hybridization
FR	family of repeats
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GC	germinal centre
GCOS	GeneChip Operating Software
GFP	green fluorescent protein
Gp	glycoprotein
GTP	guanosine triphosphate
HAT	histone acetyltransferase

HDAC	histone deacetylase complex
HIC	human I-mfa domain-containing
hIL-10	human IL-10
HINGS	heat inactivated goat serum
HIV	human immunodeficiency virus
HL	Hodgkin's Lymphoma
HLA	human leukocyte antigen
H-RS	Hodgkin Reed Sternberg
HSV	herpes simplex virus
IAP	inhibitor of apoptosis protein
IE	immediate early
IMS	intermembrane space (IMS)
INF	interferon
ITAM	immunoreceptor tyrosine-based activation motif
IVT	*in vitro* transcription
Ig	immunoglobulin
Ig_H	immunoglobulin heavy
Ig_L	immunoglobulin light
Ig_V	immunoglobulin variable
IL	interleukin
IM	infectious mononucleosis
IR	internal repeat
IRES	internal ribosome entry site
IRF	interferon regulatory factor
JAK	janus kinase
J_H	joining heavy
J_L	joining light
JNK	c-Jun N-terminal kinase
kbp	kilobase pair

kDa	kiloDalton
KO	knock out
KSHV	Kaposi's sarcoma-associated herpesvirus
LCL	lymphoblastoid cell line
LCV	lymphocryptovirus
LD	lymphocyte-depleted
LMP	latent membrane protein
LoMP	low melting point
LP	leader protein
LZ	leucine zipper
MAC	mitochondria apoptosis induced channel
MACS	magnetic activated cell sorting
MAPK	mitogen activated protein kinase
MB	Myc Box
mBL	molecular BL
MC	mixed cellularity
MHC	major histocompatibility complex
miR	microRNA
MOI	multiplicity of infection
MOM	mitochondrial outer membrane
N-terminal	amino-terminal
NFκB	nuclear factor kappa B
NGFR	nerve growth factor receptor
NK	natural killer
NLS	nuclear localisation signal
NPC	Nasopharyngeal carcinoma
NS	nodular sclerosing
NTD	N-terminal domain
ORF	open reading frames

oriP	origin of plasmid replication
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI	propidium iodide
PI3K	phosphatidylinositol 3-kinase
PML NB	promyelocytic leukemia nuclear body
PNA	peptide nucleic acid
PTLD	post-transplant lymphoproliferative disease
PKR	protein kinase R
RACK	receptor for activated C kinase
RAG	recombinase activating genes
Rb	retinoblastoma protein
RBP-Jκ	recombination binding protein J kappa
RDV	rhadinovirus
RIP	receptor interacting protein
RNA	ribonucleic acid
RT	reverse transcription
SAM	significance analysis of microarrays
SCID	severe combined immunodeficient
SHM	somatic hypermutation
SDS	sodium dodecyl sulphate
SKIP	ski-interacting protein
STAT	signalling transducer and activator of transcription
TAP	transporter associated with antigen processing
TCA	trichloroacetic acid
TEMED	N, N, N' N'-tetramethylethylenediamine
TES	transformation effector site

TK	thymidine kinase
TM	transmembrane
TNF	tumour necrosis factor
TPA	12-0-tetradecanoyl phorbol-13-acetate
TR	terminal repeats
TRADD	TNF receptor associated death domain
TRAF	TNF receptor associated factor
TRRAP	transformation/transcription domain-associated protein
TSA	trichostatin A
TSG	tumour suppressor gene
Tween	polyoxyethylenesorbitan monolaurate
UL	unique long
US	unique short
VCA	viral capsid antigen
VEGF	Vascular endothelial growth factor
V_H	variable heavy
vIL-10	viral IL-10
V_L	variable light
WHO	World Health Organisation
WT	wild type
XBP1	X-box binding protein 1
XLA	X-linked agammaglobulinemia
ZRE	Z-response elements

1. Introduction

1.1 The Discovery of Epstein-Barr virus (EBV)

The road to the discovery of the Epstein-Barr virus (EBV) began with the characterisation by Dennis Burkitt, a surgeon working in East Africa in the 1950s, of an unusual extranodal tumour in the jaws of African children (Burkitt, 1958). Burkitt Lymphoma (BL), as it later became known, was mapped by Dennis Burkitt to areas holoendemic for malaria, leading to speculation that its etiology may be linked to an arthropod-borne agent (Burkitt, 1962b; Burkitt, 1962a). To study this possibility, a collaboration was established with Tony Epstein to send BL samples to the UK for further analysis. Epstein and his then PhD student, Yvonne Barr, were able to culture a BL derived cell line (EB1) from a BL biopsy (Epstein and Barr, 1964). Analysis of EB1 by electron microscopy revealed herpes-like virus particles in some BL cells. This newly identified virus was biologically inert for all activities associated with known human herpes viruses; thus it was formally identified in 1965 as a new herpes virus (Epstein et al., 1965; Henle and Henle, 1966a; Henle and Henle, 1966b).

The establishment of BL cell lines in which a small number of cells were actively replicating the virus lead to the development of a serological assay against the viral capsid antigen (VCA). Interestingly, screening for VCA revealed that EBV was present not only in BL patients, but in 90% of the adult human population (Henle et al., 1969). It was later established that EBV was able to persist in serologically immune hosts; latent EBV infection was found in B lymphocytes and active lytic replication was observed in the oropharynx (Gerber et al., 1972). The use of VCA antiserum also identified EBV as the causative agent in the self-limiting communicable disease, infectious mononucleosis (IM) (Evans et al., 1968; Henle et al., 1968).

Importantly, it was also discovered that EBV from irradiated BL cell lines or cell free virus could be used to infect resting B lymphocytes and transform them into continuously proliferating lymphoblastoid cell lines (LCLs). This demonstrated EBV's B lymphotrophic transforming nature and established it as a ubiquitous oncogenic virus (Henle et al., 1967; Nilsson et al., 1971).

1.2 Classification of EBV

EBV (also known as human herpesvirus 4) is a member of the *Herpesviralae* family (Davison et al., 2009). This viral family is sub-divided into Alpha, Beta and Gamma members based on genome homology. All of the Gammaherpesvirinae sub-family establish latent infection in B lymphocytes and are associated with cell proliferation (Bilello et al., 2006; Marr-Belvin et al., 2008). They are further sub-divided into the gamma 1 or *lymphocryptovirus* (LCV) and the gamma 2 or *rhadinovirus* (RDV) genera. EBV is the prototype LCV and has been found exclusively in humans. The remaining LCVs are found exclusively in primates and each primate species has a closely related member of the LCV family, which is similar in both genome structure and gene organisation (Davison, 2002).

1.3 EBV structure

EBV has a toroid-shaped protein core wrapped with viral DNA (Epstein et al., 1965; Pope et al., 1968a). This is surrounded by a nucleocapsid composed of 162 capsomers, a protein tegument and an outer envelope with external glycoprotein spikes. The icosahedral EBV capsid is composed of tubular 160, 47 and 28kDa major viral proteins as well as a number of minor virion proteins. The tegument comprises a mixture of cellular and virally encoded proteins, whereas the protein content of the host derived envelope is dominated by the virally encoded 350 or 220kDa glycoprotein (gp350/220) (Dolyniuk et al., 1976a; Dolyniuk et al., 1976b; Johannsen et al., 2004).

The EBV genome is encoded within 184 kilobase pairs (kbp) of linear double-stranded DNA and contains 60% guanine/cytosine residues (Baer et al., 1984; de Jesus et al., 2003; Raab-Traub et al., 1980). It is divided into short and long, largely unique sequence domains (US and UL) by reiterated 3kbp internal direct repeats (IR1) (Hayward et al., 1980; Given and Kieff, 1979). The ends of the genome are flanked by 4-12 tandem copies of the reiterated 0.5kbp terminal repeats (TR) (Hayward and Kieff, 1977; Given et al., 1979; Kintner and Sugden, 1979), which mediate the circularisation of EBV DNA into episomes. The precise number of TRs is determined during viral replication and can serve as a useful indicator of cell clonality, as EBV in latently infected progeny cells retains the same number of TRs as in the parental cell (Raab-Traub and Flynn, 1986).

The B95-8 EBV strain was the first herpes virus to have its genome fully cloned and sequenced (Dambaugh et al., 1980; Arrand et al., 1981; Baer et al., 1984; Hatfull et al., 1988; de Jesus et al., 2003). A schematic map of the EBV genome is shown in Figure 1.1. The initial sequencing was carried out on a *Bam*HI fragment cloned library, hence open reading frames (ORFs), genes, or sites of viral RNA transcription are frequently referenced to specific *Bam*HI fragments. Thus the EBV polymerase gene is frequently referred to as BALF5 for *Bam*HI, A fragment, fifth leftward ORF. Sequencing of the EBV genome also revealed several viral homologues of human cellular genes, including homologues to the *jun/fos* family, Bcl-2 and interleukin-10 (IL-10).

There are 2 major subtypes of EBV, currently referred to as type 1 and type 2, which can be further subdivided into different EBV strains (Sixbey et al., 1989). All EBV isolates are highly homologous and share a common genome organisation (Nonoyama and Pagano, 1973; Pritchett et al., 1976). The sequence of type 1 and type 2 viruses only differs significantly in the genes which encode the Epstein-Barr nuclear antigens (EBNA) 2, 3A, 3B and 3C (McGeoch and Gatherer, 2007). Sequence variation between these 2 subtypes results in a predicted difference in the primary amino acid (aa) sequence of EBNA 2, 3A, 3B and 3C of 47%, 16%, 20% and 28% respectively (Sample et al., 1990). EBV strains of the same type can be distinguished according to changes in polymorphic sites, such as the number of repeated sequences in several latently expressed genes, single nucleotide changes in the genes encoding EBNA1, EBNA2 and LMP1 and the presence or absence of a 30bp region within the LMP1 gene (Hennessy and Kieff, 1983; Lung et al., 1990; Hu et al., 1991; Chen et al., 1992b; Lung and Chang, 1992; Lung et al., 1994; Miller et al., 1994b; Falk et al., 1995; Khanim et al., 1996; Sandvej et al., 1997). It is believed that these polymorphic changes may have occurred due to a slow evolutionary drift in geographically separate human populations; however the origin of the two virus subtypes remains enigmatic as both subtypes are found in the oropharynx of most human populations. Type 1 EBV is epidemiologically dominant except in areas endemic for BL (equatorial Africa and New Guinea) where infection with type 2 is almost as common (Sixbey et al., 1989; Sculley et al., 1988; Young et al., 1987).

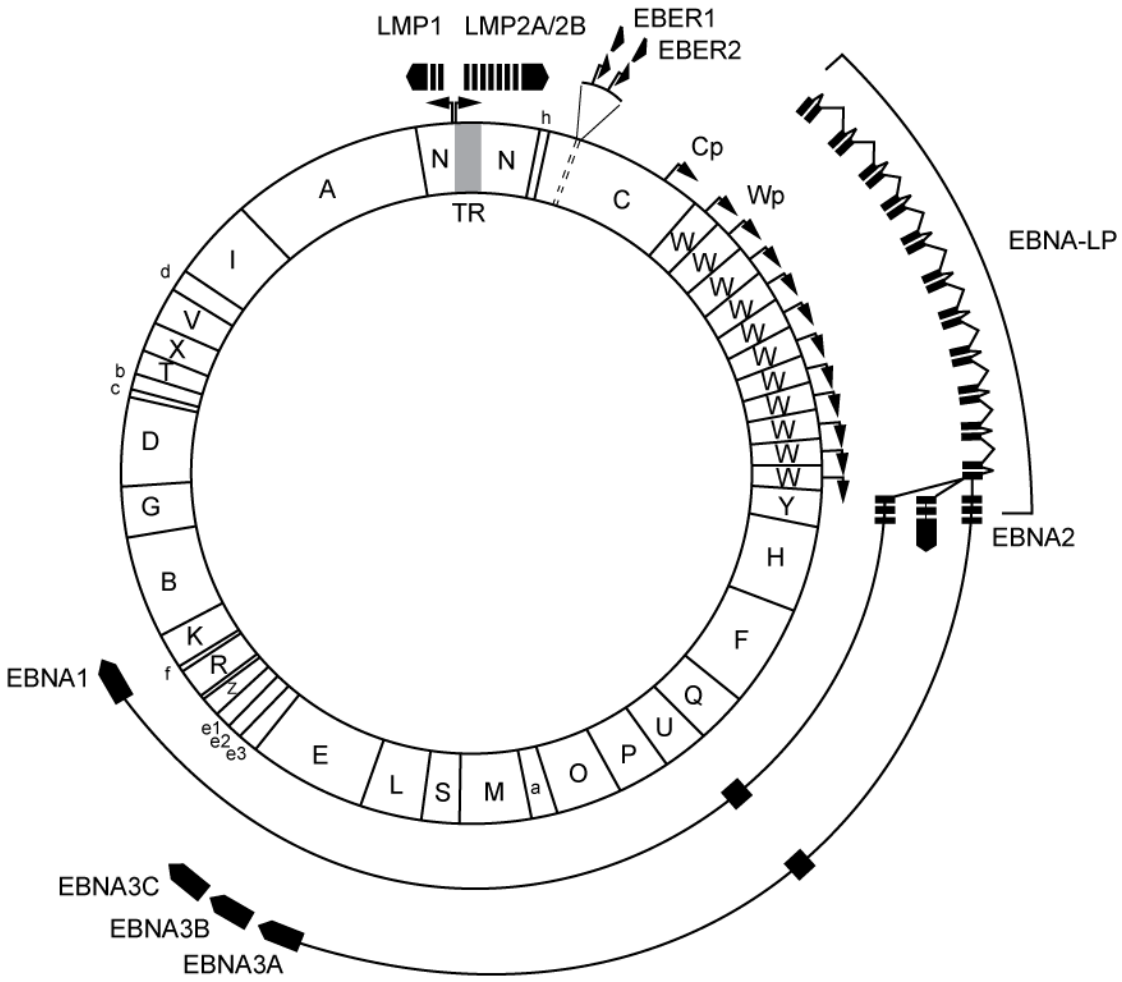


Figure 1.1. Schematic map of the EBV genome divided into *Bam*HI digest fragments. Arrows represent latent promoters, filled boxes represent genes encoding the latent proteins and EBERs and the grey box represents the terminal repeats (TRs)

1.4 EBV Tissue Tropism

1.4.1 Infection *in vivo*

It is now well established that EBV resides as a latent infection within the memory B cell pool of infected healthy carriers (Babcock et al., 1999). Lytic EBV infection is also believed to occur within B cells (Anagnostopoulos et al., 1995); however there is also evidence of rare lytic replication in mucosal epithelial cells (Herrmann et al., 2002). In addition, we know from the discovery of EBV in a broad spectrum of malignancies that EBV is capable of infecting other cell types. Along with its association with B cell tumours such as BL, EBV has been associated with T and NK lymphomas (Harabuchi et al., 1990), smooth muscle cell tumours (Lee et al., 1995) and a number of epithelial carcinomas (Greenspan et al., 1985; Wolf et al., 1973; Wong et al., 1995).

1.4.2 Infection *in vitro*

Critical to understanding the role of EBV as an oncogenic virus was the seminal discovery that it was able to infect and transform primary B cells *in vitro* (Henle et al., 1967; Pope et al., 1968b). Infection resulted in the conversion of primary B cells to continuously proliferating lymphoblastoid cell lines (LCLs), where most cells are non-permissive for viral replication. Subsequently EBV was found to readily infect B lymphocytes derived from peripheral blood, tonsils or foetal cord blood (Anvret and Miller, 1981; Henle et al., 1967; Henderson et al., 1977; Pope et al., 1968b; Zerbini and Ernberg, 1983). Bone marrow or liver B lymphocytes from earlier stages of development, as well as leukemic and non-EBV-infected BL cell lines can also be infected with EBV. The efficiency of infection is however lower than in peripheral blood B cells and EBV is unable to infect fully differentiated plasma cells (Avila-Carino et al., 1994; Calender et al., 1987; Hansson et al., 1983). *In vitro* EBV has also been shown to infect cells from non-B cell lineages including primary T cells, natural killer (NK) cells and epithelial cells; however infection efficiency with cell-free virus is very low compared to B lymphocyte infection (Kanegane et al., 1996; Shapiro et al., 1982; Sixbey et al., 1983; Young et al., 1986). Interestingly, infection of epithelial cells can be significantly improved through coculture with B cells with surface bound EBV particles (Pegtel et al., 2004; Shannon-Lowe et al., 2006), possibly reflecting a novel route of infection *in vivo*.

To obtain new virus isolates, EBV transformed cell lines can be generated by incubation of throat washing from EBV-infected individuals with human B lymphocytes. Alternatively, because 1 in 10^5 to 10^6 peripheral blood lymphocytes are infected with EBV, culture of 10^6 to 10^7 B lymphocytes from a seropositive individual in the absence of functional T lymphocytes is likely to result in the outgrowth of EBV-infected B lymphocytes, even without the addition of exogenous virus (Nilsson et al., 1971; Rickinson et al., 1977). Virtually all cell lines that arise from cultures of normal human blood cells are EBV immortalised B cell derived LCLs; however recovery of LCLs from EBV-infected cultures can be blocked by the addition of neutralizing antibodies to EBV (Rickinson and Epstein, 1978; Thorley-Lawson and Strominger, 1976). The rationale is that explantation into culture can apparently cause a rare EBV-infected cell to become permissive for viral infection and an immortalised cell line then results from latent infection of surrounding B cells.

EBV infection of primary B cells *in vitro* results in around 10% of cells becoming latently infected and proliferating as transformed LCLs (Henderson et al., 1977), although this is dependent on the multiplicity of infection (MOI) used (Shannon-Lowe et al., 2005). Latently infected B cells express 6 EBV nuclear antigens (EBNAs), EBNA 1, 2, 3A, 3B, 3C and LP and 3 latent membrane proteins (LMPs), 1, 2A and 2B. They also express the small, non-polyadenylated, non-coding, but highly expressed EBER1 and EBER2 transcripts, the highly spliced *Bam*HI-A rightward transcripts (BARTs) and a number of EBV-encoded microRNAs (miRs). Expression of these viral gene products immediately and efficiently causes previously resting B lymphocytes to continuously proliferate, with most cells entering DNA synthesis 48-72 hours after EBV infection. These proliferating B cells resemble lymphocytes proliferating in response to antigen, mitogen, or stimulation with CD40 and IL4; they express a similar repertoire of activation-associated proteins, secrete immunoglobulin (Ig) and adhere to one another (Banchereau et al., 1991; Bonnefoy et al., 1988; Sugden and Metzenberg, 1983; Wang et al., 1988; Zhang et al., 1991). During infection of primary B cells the target cell for EBV infection is usually a resting B cell (Aman et al., 1984). However, as previously mentioned, most EBV-negative BL cells (which proliferate due to deregulated c-Myc expression) can also be infected with EBV, resulting in the same programme of latency as observed in LCLs (Calender et al., 1987; Calender et al., 1990). Most infected BL cells continue to replicate the EBV genome episomally;

however in some cases it becomes integrated into the cellular genome (Henderson et al., 1983; Lawrence et al., 1988).

1.5 Events during primary infection of B cells *in vitro*

The initial events of EBV infection of B cells involve binding to the EBV receptor, internalisation and genome circularisation within the nucleus of the infected cell. EBV efficiently binds to the innate immune system C3d complement receptor, CD21 on the surface of B lymphocytes via its most abundant glycoprotein, gp350/220 (Fingeroth et al., 1984; Nemerow et al., 1987; Lambris et al., 1985). Expression of CD21 during B-lymphocyte development correlates with the efficiency of viral binding and EBV infection can be blocked by monoclonal CD21 antibodies, purified CD21 or by saturation of B-lymphocyte receptors with gp350/220 (Jondal et al., 1976; Tanner et al., 1988). Binding of gp350/220 triggers capping of CD21 and endocytosis of the virus into cytoplasmic vesicles (Nemerow and Cooper, 1984). CD21 aggregation by EBV may also result in CD21 tyrosine kinase signal transduction. This may have an activating effect on the infected B-lymphocyte as soon after infection the cells enlarge, synthesize RNA, express activation and adhesion markers, clump together and begin to secrete Ig (Martin et al., 1994; Masucci et al., 1987). CD21 ligation also increases NFκB activation, which may up-regulate the EBV W promoter (Wp) and induce interleukin-6 expression via a protein kinase C pathway (D'Addario et al., 2001; Sugano et al., 1997).

Although gp350/220 represents EBV's primary mechanism for B cell binding, a gp350/220 knock out (KO) recombinant EBV is still able to transform B cells, albeit at a much-reduced efficiency (Janz et al., 2000). B lymphocyte binding of EBV lacking gp350/220 is likely to be via EBV's second most abundant viral envelope protein, the viral glycoprotein H (gH) homologue, gp85. Gp85 forms a heterodimer with the EBV gL homologue, gp25 and a heterotrimeric complex with gp25 and gp42 (Heineman et al., 1988; Edson and Thorley-Lawson, 1983). The heterotrimeric gp85/gp25/gp42 complex acts as a coreceptor for B lymphocyte internalisation of EBV by engagement of the human leukocyte (HLA) class II complex, whereas the gp85/gp25 heterodimeric complex is believed to play a role in binding to CD21 surface negative epithelial cells through binding to an as yet unidentified epithelial surface receptor (Molesworth et al., 2000; Oda et al., 2000b). Interestingly, EBV shed from epithelial cells is rich in gp42, thus increasing the presence of heterotrimeric gp85/gp25/gp42

complexes and depleting the plasma membrane of the gp85/gp25 heterodimer. In B cell produced virus the opposite is true; low gp42 on the producer cells results in dominance of the gp85/gp25 heterodimer. As a result, epithelial cell generated virus is as much as two orders of magnitude more infectious for B lymphocytes than virus produced from B cells, whereas B cell produced virus more efficiently infects epithelial cells. This suggests a model of EBV persistence where virus reactivating from a latently infected memory B cell is equipped to infect an epithelial cell, whereas virus amplified in an epithelial cell is strongly B cell tropic and so much more likely to go on to infect a resting B cell (Borza and Hutt-Fletcher, 2002).

Although little is known about EBV capsid dissolution, genome transport to the nucleus and DNA circularization, it is likely, by analogy to other DNA viruses, that EBV capsid transport to nuclear pores is mediated by the cytoskeleton (Dales and Chardonnet, 1973). Infection is completed by the covalent closure of the linear viral DNA to form the circular EBV genomes observed in virally infected cells (Adams and Lindahl, 1975; Lindahl et al., 1976).

1.6 Latent infection in transformed primary B cells

EBV infection of primary B cells *in vitro* results in a pattern of viral gene expression termed 'Latency III'. Circularized viral genomes appear within the nuclei of infected cells 12 to 16 hours after virus binding. At around the same time, the W promoter (Wp), present in the 3kbp, *Bam*HI-W or IR1, long internal repeat element initiates rightward transcription (Alfieri et al., 1991; Moss et al., 1981). The B cell specificity of this promoter is in part mediated by B cell lineage specific activator protein (BSAP/Pax5) binding sites upstream of Wp (Tierney et al., 2000; Tierney et al., 2007).

Wp promotes the transcription of the first EBV RNAs, which are differentially spliced to generate mRNAs coding for the EBNA-LP and EBNA2 proteins. These transcripts are transcribed by host cell RNA polymerase II as EBV does not encode its own polymerase enzymes. Figure 1.2 is adapted from (Qu and Rowe, 1992) and shows the differential splicing from Cp and Wp. The initial Wp driven mRNAs have a 40 bp first exon, W_0, which ends in an AT (Alfieri et al., 1991). To generate EBNA-LP transcripts, W_0 is spliced on to the 5bp truncated W_1 exon, W_1'. W_1' begins with a G residue, thus creating an ATG translational start site. The W_1' exon is followed by a 132 base W_2 exon, then by 2-10 repeating 66 base and 132 base W_1W_2 exons derived from successive reiterations of the long internal

repeats. The EBNA-LP transcript ends in unique *Bam*HI Y exons, Y_1 and Y_2. EBNA2 transcripts initiated from Wp splice from the W_0 exon to the un-truncated W_1 exon, thus ablating the ATG translational start site of EBNA-LP. EBNA2 transcripts then splice into W_2, through the multiple W_1 and W_2 exons and into the EBNA2 ORF, BYRF2 (Speck and Strominger, 1985; Sample et al., 1991; Wang et al., 1991).

EBNA-LP and EBNA2 protein reaches the level maintained in LCLs 24 to 32 hours after infection (Alfieri et al., 1991; Cordier et al., 1990; Knutson, 1990; Zimber-Strobl et al., 1993). Once expressed, EBNA-LP and EBNA2 protein coordinate to up-regulate transcription from the upstream *Bam*HI C promoter (Cp) (Bodescot et al., 1987; Evans et al., 1996; Jin and Speck, 1992). In those cells where Cp is switched on, it supplants Wp as the dominant promoter for transcription of the EBNAs (Woisetschlaeger et al., 1989). Two short exons downstream of Cp, C_1 and C_2, replace the short W_0 exon in the EBNA mRNA and, as with Wp initiated transcripts, alternative splicing results in mRNAs incapable or capable of translating EBNA-LP (Speck et al., 1989). The switch from Wp to Cp also leads to the expression of the remaining EBNA transcripts (Speck and Strominger, 1985). In order to generate the EBNA3s and EBNA1 transcripts, a splice donor near the beginning of the YH exon is spliced to a downstream acceptor in *Bam*HI-U (Bodescot and Perricaudet, 1986; Rogers et al., 1990). This U exon is then spliced into any of 4 alternative acceptor sites that begin the ORFs that encode the amino terminus of EBNA3A, 3B and 3C or EBNA1, each of which encodes its own polyadenylation signal. Expression of these EBNAs is detectable 20-32 hours after infection and peaks within 48-72 hours. Expression of EBNA1 further promotes EBNA expression through EBNA1 binding to the EBNA1-dependent enhancer component of oriP located 3.5kbp upstream of Cp (Gahn and Sugden, 1995; Puglielli et al., 1996; Reisman and Sugden, 1986). In LCLs the frequency of each EBNA transcript is determined by alternative acceptor site usage and efficient translation is aided by an internal ribosome acceptor site (IRES) (Isaksson et al., 2003). This positive feedback on Cp activity initially mediated by EBNA2 and EBNA-LP and then EBNA1 is balanced by a repressive action of the EBNA3 proteins (Johannsen et al., 1996; Marshall and Sample, 1995a).

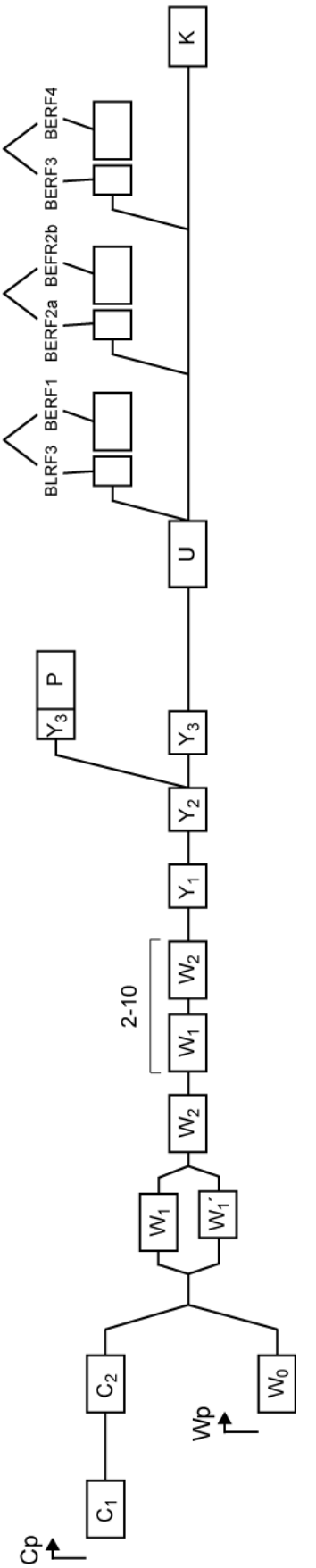


Figure 1.2. This figure is adapted from Qu and Rowe, 1992 and shows differential splicing of EBV latent viral transcripts in an EBV transformed B lymphocyte. Open boxes represent open reading frames (ORFs) and arrows represent the viral promoters, Cp, Wp, Qp and Fp.

As previously mentioned, LMP expression is activated by EBNA2 and EBNA-LP. LMP2A and 2B transcription is in the same direction as the EBNAs and their expression is regulated through EBNA2 response elements upstream of their promoters (Zimber-Strobl et al., 1993). LMP1 is transcribed in the opposite direction and lies entirely within the antisense strand of the first intron of LMP2A. It has two independent promoters, one which lies in the same upstream regulatory sequence as the LMP2B promoter (Fennewald et al., 1984; Sjoblom et al., 1998) and a second less well characterised promoter located in the TR region which is activated independently of EBNA2 in some EBV-associated malignancies (Sadler and Raab-Traub, 1995a). By 32 hours after infection of B lymphocytes, all EBNA and LMP mRNAs can be detected and by 48 hours expression reaches a level close to that observed in an established LCL (Alfieri et al., 1991; Allday et al., 1989; Moss et al., 1981). As discussed in section 1.8.5, expression of LMP1 has a dramatic impact on the phenotype of an infected cell; it initiates DNA synthesis and up-regulates adhesion and activation markers and anti-apoptotic proteins (Peng and Lundgren, 1992; Peng and Lundgren, 1993; Fries et al., 1996).

The last of the EBV latent transcripts to be expressed are the non-coding, non-polyadenylated EBERs. 70 hours after the initial infection, EBER1 and EBER2 RNAs reach substantial expression levels and, in established LCLs, EBERs are the most abundantly expressed EBV RNAs (King et al., 1980; Sample et al., 1992). EBERs are transcribed predominantly by cellular RNA polymerase III, although polymerase II may also be involved (Arrand and Rymo, 1982; Howe and Shu, 1989). They have typical intragenic control regions common to pol III transcripts and upstream Sp1, ATF and TATA box elements characteristic of pol II transcription sites (Howe and Shu, 1993).

1.7 Alternate patterns of latent infection

Figure 1.3 is adapted from Fields et al., 2001 and shows the four different patterns of viral gene expression observed in latent EBV infection.

1.7.1 Latency III

The pattern of latent antigen expression and viral promoter usage observed in EBV transformed LCLs is referred to as Latency III. Cells constitutively express the six EBV nuclear antigens, EBNA1, 2, 3A, 3B, 3C and LP, from large highly spliced transcripts initiated from Cp or Wp. LCLs also express the

latent membrane proteins, LMP1, 2A and 2B, non-coding EBER transcripts, the BARTs (Alfieri et al., 1991; Brooks et al., 1993) and a number of EBV encoded miRs from their own promoters (Cai et al., 2006). B cells carrying EBV in a Latency III infection acquire a lymphoblastoid phenotype which resembles that of B cells *in vitro* stimulated with mitogen/antigen; they up-regulate activation and adhesion markers, HLA molecules and acquire efficient antigen processing (Wang et al., 1988; Wang et al., 1990; Peng and Lundgren, 1993). *In vivo*, Latency III transcription is rarely observed in immunocompetent EBV carrying individuals because of strong immunosurveillance by CD8+ cytotoxic T lymphocytes (CTLs). However, Cp and Wp initiated transcripts can be found in peripheral blood mononuclear cells (PBMCs) from patients with infectious mononucleosis (IM), indicating that *in vivo*, primary infection is associated with a transient virus-driven expansion of the infected B-cell pool through Latency III viral gene expression (Tierney et al., 1994). When this immunosurveillance by CD8+ CTLs is compromised, for example by immunosuppressive drugs in post-transplant patients, virus infected B cells can be spontaneously transformed by EBV causing post-transplant lymphoproliferative disease (PTLD) (Young et al., 1989; Gratama et al., 1991).

1.7.2 Latency I

In contrast to the full Latency III infection observed in LCLs and PTLD, restricted Latency I viral gene expression is characterised by activity of the novel Q promoter (Qp) and silence of Cp, Wp, and the LMP promoters (Schaefer et al., 1995b; Nonkwelo et al., 1996). Spliced Q-U-K transcripts from Qp lead to expression of only the EBNA1 protein, while separate promoters initiate high level expression of the non-coding EBER transcripts, the BARTs (Rowe et al., 1987; Brooks et al., 1993) and the EBV-encoded miRs (Cai et al., 2006). Latency I viral gene expression is observed in the B cell derived tumour, Burkitt Lymphoma (BL) and most early passage BL derived cell lines (Rowe et al., 1987).

EBV-positive BL cells are phenotypically distinct from cells with a Latency III infection; instead of the adhesion and activation markers observed in LCLs, they express germinal centre (GC) markers CD10 and CD77. BL cells also lack many of the components of the HLA class I antigen processing pathway, apparently aiding them to escape immune detection by CD8+ CTLs (Masucci, 1990; Khanna et al., 1994; Frisan et al., 1996). During serial passage in culture *in vitro* some BL cells are unable to maintain Qp driven Latency I viral gene expression and spontaneously drift to the full Cp/Wp using

Latency III expression observed in LCLs. This change is accompanied by the acquisition of an LCL-like cellular phenotype and an increase in resistance to apoptosis (Gregory et al., 1991; Rooney et al., 1986; Rowe et al., 1987).

Recently, a novel form of viral gene expression has been described in a subset of BL tumours (Kelly et al., 2002; Kelly et al., 2006). Termed 'Wp restricted tumours' because of the activation of only Wp, viral gene expression from these cells originates from an EBNA2 deleted genome. These tumours express EBNA 1, 3A, 3B, 3C, and a truncated EBNA-LP, along with the non-coding EBERs and BARTs.

1.7.3 Latency II

Latency II is another form of restricted viral latency. As observed in Latency I, EBNA1 is expressed from Qp initiated Q-U-K transcripts along with the EBERs, BARTs and miRs. However Latency II infected cells also express variable levels of LMP1, LMP2A and LMP2B from alternative EBNA2-independent promoters located in the *Bam*HI N region of the genome (Brooks et al., 1992; Brooks et al., 1993; Kerr et al., 1992; Deacon et al., 1993). This form of infection was initially identified at the protein level in biopsies of the epithelial cell tumour, nasopharyngeal carcinoma (NPC) and was subsequently also found in the Reed-Sternberg cells of EBV-positive Hodgkin's Lymphoma (HL) (Fahraeus et al., 1988; Young et al., 1988).

1.7.4 Latency 0

It is believed that EBV may persist in memory B cells in a fifth form of viral latency, termed Latency 0, where all latent gene expression, with the possible exception of the EBERs and BARTs, is suppressed (Babcock et al., 1999). Reactivation of EBV latent transcription from this pool of infected cells may account for the low level LMP1, LMP2A, and Qp-initiated EBNA1 mRNA transcripts present in the GC B and memory B cell compartments of tonsillar lymphocytes (Babcock and Thorley-Lawson, 2000).

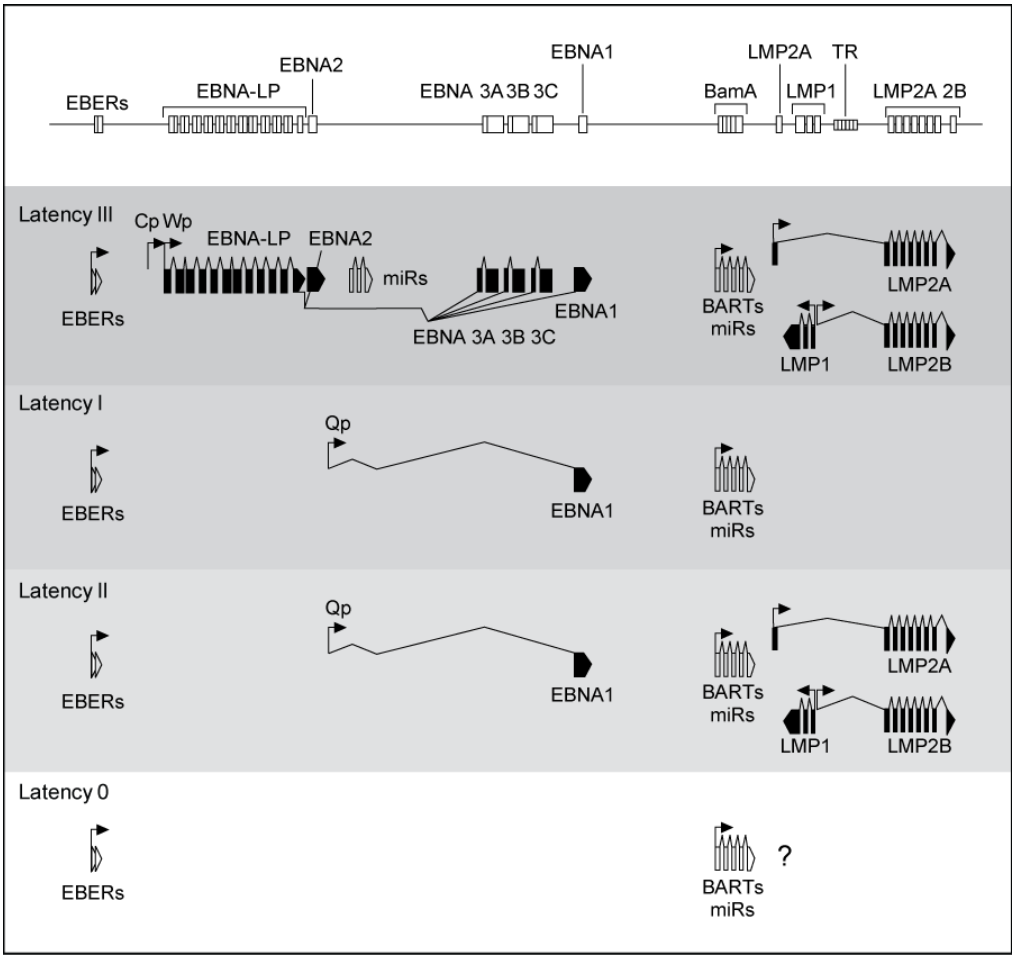


Figure 1.3. This figure is adapted from Fields et al., 2001 and shows a schematic diagram of EBV latent viral transcription in the different forms of latency. Boxes represent EBV latent ORFs; shaded boxes represent ORFs which are translated into viral protein, whereas open boxes represent non-coding viral RNAs. A linear EBV genome is shown above with the relative positions of the latent genes and the terminal repeats (TR) marked.

1.8 EBV latent gene products

1.8.1 EBNA1

EBNA1 is a nuclear antigen encoded by the EBV BKRF1 ORF. It is the most ubiquitously expressed of the EBV encoded proteins and is found in all forms of viral infection, with the exception of Latency 0 (Reedman and Klein, 1973; Babcock et al., 1999). In Latency III, EBNA1 mRNA is generated by heavy splicing of large Cp or Wp initiated primary transcripts (Bodescot et al., 1986; Sample et al., 1991; Speck and Strominger, 1985), while In Latency I and Latency II infections, EBNA1 mRNA transcripts are initiated at the alternative *Bam*HI Q promoter. EBNA1 is also the only latent antigen to be expressed during the lytic cycle; lytic EBNA1 mRNAs initiate directly from the lytic F promoter (Fp), located just upstream of Qp.

The EBNA1 protein encoded by the prototype B95-8 EBV strain consists of 641 amino acids. It has high proline content, is positively charged and migrates to an apparent size of 76kDa on a denaturing polyacrylamide gel. Figure 1.4 shows the position of the major functional domains within the EBNA1 protein. The full length protein has linkage regions, LR1 and LR2, at aa positions 40-89 and 327-377, which flank a Glycine-Alanine (Gly-Ala) repeat region. This is followed by a nuclear localisation sequence located at position 377-386, a DNA-binding and dimerisation domain at position 451-607 and a C-terminal acidic activating domain at position 607-641.

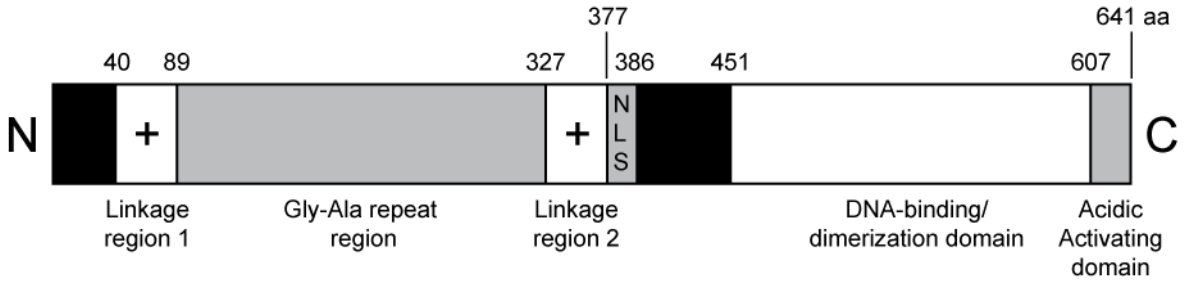


Figure 1.4. Schematic representation of the major domains of the EBV nuclear antigen 1. Amino acid reference numbers refer to the position on the protein encoded by the B95-8 EBV strain, NLS refers to the nuclear localisation signal and N and C mark the amino and carboxyl termini.

EBNA1 is not essential for B lymphocyte transformation by EBV; however its deletion reduces transformation efficiency around 1000-fold (Humme et al., 2003). This reduction in transformation

efficiency results from an inability of infected cells to maintain an episomal viral infection. EBNA1 episomally maintains EBV genomes through diffuse association with mitotic chromosomes (Ohno et al., 1977), a property that is unique among the EBNAs (Petti et al., 1990). It also binds strongly and specifically to 30bp repeats within cis acting elements of the EBV origin of plasmid replication (oriP) (Rawlins et al., 1985; Yates et al., 1984). OriP has 2 major components: an EBNA1-dependent enhancer (FR), composed of a family of 20 copies of the 30bp repeat, and a dyad symmetry (DS) component, composed of four of the 30bp repeats (two in tandem and two in a larger dyad symmetry) (Reisman et al., 1985).

FR is responsible for the EBV-mediated up-regulation of Cp and Wp and has also been shown to exert a long range effect on the LMP genes (Gahn and Sugden, 1995; Puglielli et al., 1996; Reisman and Sugden, 1986). Binding of EBNA1 to the DS region is responsible for genome segregation. The association between chromosomal DNA, EBNA1 and oriP mediates a single replication of the EBV genome during S phase, followed by equal segregation of replicated EBV genomes to progeny cells during cell division (Adams, 1987; Yates and Guan, 1991). EBNA1 has also been identified as a possible enhancer of cell survival (Kennedy et al., 2003), possibly through its ability to bind the p53 binding protein, ubiquitin specific protease 7 (USP7) (Saridakis et al., 2005). The possible protective role of EBNA1, especially in the context of BL, is discussed further in section 1.15.1.

Column chromatographic, cross-linking and motility retardation assays suggest that EBNA1 exists as a dimer, both in solution and when bound to DNA (Ambinder et al., 1991). It is believed that this is a function of the overlapping dimerisation and DNA binding domains, which in isolation have similar binding and dimerisation properties to the full length EBNA1 protein (Ambinder et al., 1991; Jones et al., 1989; Rawlins et al., 1985). The DNA binding/dimerisation domain appears to be responsible for the initial accumulation of oriP containing plasmids in cells; however it is insufficient to support long term episomal replication, without the presence of at least one of the functionally redundant, arginine and glycine rich linkage regions (Mackey and Sugden, 1999). The length of the Gly-Ala repeat region varies significantly among different EBV isolates and has been implicated in EBNA1's ability to evade the host immune response by inhibiting proteasomal degradation and major histocompatibility complex (MHC) presentation.

Expression of EBNA1 is up-regulated by a number of cellular factors, including the interferon regulatory factors, IRF1 and 2 and the cell cycle E2F transcription factors, all of which bind to three distinct regions that bracket the EBNA1 transcription initiation site (Nonkwelo et al., 1997; Schaefer et al., 1997). In Latency I and Latency II, EBNA1 transcription is limited through binding of EBNA1 to binding sites just to the 3' of the transcriptional start site.

1.8.2 EBNA2

EBNA2 is encoded by the BYRF1 ORF and is one of the first viral proteins to be expressed during transformation of primary B cells. Initially expressed from Wp, EBNA2 and EBNA-LP activate the adjacent Cp promoter (Bodescot et al., 1987; Evans et al., 1996; Jin and Speck, 1992), which then becomes the dominant promoter for expression of all six EBNAs (Speck and Strominger, 1985; Woisetschlaeger et al., 1989). Deletion of the EBNA2 gene and the last 2 exons of EBNA-LP in the transformation incompetent P3HR-1 virus strain provided the first indication of the critical role of the EBNA2 protein in the transformation process (Dambaugh et al., 1984; Nagoya and Hinuma, 1972; Rabson et al., 1982; Skare et al., 1985). Restoration of the deleted DNA segment by homologous recombination unequivocally confirmed the importance of EBNA2 in B cell transformation and allowed the identification of the functionally relevant domains (Cohen et al., 1989; Hammerschmidt and Sugden, 1989; Marchini et al., 1992).

EBNA2 functions as a potent and specific activator of viral and cellular gene expression. It contains a number of distinct structural elements: 1) a negatively charged 57 aa N-terminal domain, 2) a variable polyproline repeat region, flanked by a pair of homotypic association domains, 3) a highly divergent central region, 4) an RBP-Jκ interacting region, 5) an imperfect Arg-Gly repeat that can provide a nuclear localization signal (NLS), 6) a negatively charged region containing a transactivation domain and 7) a C-terminal region, which contains a Lys-Arg-Pro-Arg repeat and provides a second NLS. Of these structures, only the N-terminal and imperfect Arg-Gly regions are highly conserved between type 1 and type 2 viruses. The overall predicted primary aa sequences of type 1 and type 2 EBNA2 differ by 47% (Sample et al., 1990) and this difference is believed to account for the greater transformation efficiency of type 1 viruses (Rickinson et al., 1987; Aitken et al., 1994; Lucchesi et al., 2008).

Three of EBNA2's structural elements, the RBP-Jκ binding domain, the acidic activation domain and the homotypic association domains have been identified as essential to the transformation and transcriptional properties of EBNA2. The RBP-Jκ binding domain lies between aa residues 290-337 and appears to mediate promoter modulation in a similar manner to the critical developmental protein, Notch 2 (Hofelmayr et al., 1999). RBP-Jκ, either alone or in concert with Ski-interacting protein (SKIP), tethers EBNA2 to its response elements upstream of cellular and viral promoters (Grossman et al., 1994; Henkel et al., 1994; Waltzer et al., 1994; Zimber-Strobl et al., 1994; Zhou et al., 2000). In LCLs, RBP-Jκ has been implicated in the EBNA2-mediated regulation of Cp (Bodescot et al., 1987; Evans et al., 1996), LMP1 (Abbot et al., 1990; Fahraeus et al., 1990; Wang et al., 1990), LMP2A and LMP2B (ZimberStrobl et al., 1993) and cellular genes, CD21 (Aman et al., 1990; Cordier et al., 1990), CD23 (Wang et al., 1987) and c-Fgr (Knutson, 1990).

The acidic activation domain lies between aa 420-464 and contains a sequence similar to the herpes simplex virus (HSV)-encoded transcriptional activator protein, VP16. EBNA2 shares affinity with VP16 for a range of transcriptional activators including p300/CBP (Grundhoff et al., 1999; Wang et al., 2000a) and has around 100 times greater affinity than VP16 for the scaffolding protein, p100. In LCLs, the acidic domain of EBNA2 is highly complexed with p100 and this is believed to play an important role in EBNA2-mediated transcription through interaction with c-Myb, PIM-1 and TFIIE. Association of the acidic domain with p300 and CBP is also believed to be critical for EBNA2-mediated transcription through their ability to mediate histone acetylation and because of their association with other transcription factors. CBP has been implicated in EBNA2 activation of c-Myc while p300 appears primarily to have a repressive role (Jayachandra et al., 1999; Wang et al., 2000b).

Finally, either of the regions bordering the polyproline repeat appears to mediate the homotypic association pivotal to EBNA2's ability to form complexes than can recruit transcription factors (Harada et al., 2001). There appears to be a degree of redundancy, as deletion of a single one of these regions causes only a moderate loss of primary B cell transformation and Cp activation; however loss of both regions completely ablates transformation (Yalamanchili et al., 1996).

1.8.3 EBNA-LP

EBNA-LP is transcribed from Cp or Wp initiated C_1 C_2 or W_0 exons followed by a variable number of W_2 W_1 repeats and unique Y_1 and Y_2 exons (Figure 1.2). Along with EBNA2, it is the first latent protein to be expressed during EBV transformation of B lymphocytes (Alfieri et al., 1991). The size of the EBNA-LP protein varies between different EBV isolates because of variation in the number of the 66 aa W_2 W_1 encoded regions (Dillner et al., 1986; Finke et al., 1987). EBNA-LP is phosphorylated at multiple sites by p34cdc2 and casein kinase II and phosphorylation is greatest during the G2/M growth phase (Kitay and Rowe, 1996; Petti and Kieff, 1988). The 44 aa W_2 encoded repeats have two stretches of basic amino acids, which provide a nuclear localization sequence for EBNA-LP (Peng et al., 2000). Within the nucleus, it associates with the nuclear matrix fraction (Petti and Kieff, 1988). Some EBNA-LP localizes to nuclear dots that correspond to sites of ND10 proteins and EBV IR1 DNA, suggesting a role in RNA processing of nascent EBNA transcripts (Szekely et al., 1996). *In vitro* biochemical studies have also suggested a possible interaction of EBNA-LP with both p53 and Rb; however these interactions have not been demonstrated in LCLs so their significance remains unclear. EBNA-LP appears to be important, but not essential for B cell transformation. A stop codon mutation, which prevents expression of the last 45 aa of EBNA-LP reduced EBV transformation of B lymphocytes and retarded the growth of the resulting LCLs (Mannick et al., 1991). However, a mutant lacking the full EBNA-LP ORF is required to fully assess its role in transformation.

The major role of EBNA-LP is to specifically potentiate the effect of EBNA2-mediated transcriptional regulation. Transient transfection of EBNA-LP and EBNA2 into primary B cells induced transition of cells from G0 to G1, as determined by up-regulation of cyclin D2 expression (Sinclair et al., 1994). In reporter constructs assays, EBNA-LP also coactivated the EBNA2-mediated up-regulation of Cp and LMP1. Most of this activity can be attributed to an indirect interaction of the W_2 W_1 repeat region with the acid activation domain of EBNA2 (Harada and Kieff, 1997; Nitsche et al., 1997; Peng et al., 2004; Peng et al., 2000), which is believed in part to be mediated through binding of EBNA-LP to the promyelocytic leukemia nuclear body (PML NB)-associated protein, Sp100 (Ling et al., 2005).

The unique 3' exons of EBNA-LP encode 11 and 34 aa C-terminal domains, which appear to regulate the activity of the repeat region. Addition of up to the first 35 C-terminal residues nearly completely

abolishes the coactivation ability of the W_1, W_2 repeat region, whereas addition of the entire 45 aa C-terminus restores wild type (WT) coactivation (Mannick et al., 1991; McCann et al., 2001).

1.8.4 The EBNA3 family

The EBNA3 genes are tandemly arranged within the genome and are transcribed from the far upstream C or W promoters. The EBNA3A, 3B and 3C ORFs each consist of a unique short 5' exon (BLRF3, BERF2a or BERF3) and a long 3' exon (BERF1, BEFR2b or BERF4) (Joab et al., 1987; Ricksten et al., 1988). The EBNA3A, 3B and 3C proteins all have repeating C-terminal polypeptide domains and share distant homology within the 5' and 3' exons implying amplification from a common cellular progenitor gene (Baer et al., 1984; Hennessy and Kieff, 1985; Kallin et al., 1986; Petti and Kieff, 1988; Petti et al., 1988). Type 1 and type 2 EBNA3A, 3B and 3C share only 84%, 80% and 72% aa sequence identity; however, unlike in EBNA2, variation of EBNA 3 alleles does not appear to affect transformation efficiency (Tomkinson and Kieff, 1992a). Each of these large multifunctional proteins has a highly charged N terminus, clusters of arginines or lysines, which may provide a nuclear localization signal and heptad repeats of leucine, isoleucine or valine that can act as dimerisation domains. Despite expression of only a few copies of EBNA3 mRNA transcripts per infected cell, the stability of the EBNA3 proteins leads to their accumulation in intranuclear clumps, which spare the nucleolus (Hennessy and Kieff, 1985; Petti and Kieff, 1988; Petti et al., 1988). Degradation of the EBNA3s leads to the presentation of highly immunogenic peptides by MHC1 on the surface of EBV-infected cells, which provide a major target for CD8+ CTLs (Murray et al., 1992; Tamaki et al., 1995).
EBNA3A and EBNA3C are critical for B cell transformation, while EBNA3B is dispensable (Tomkinson and Kieff, 1992b). However, the fact that EBNA3B is retained within virus populations, despite being highly immunogenic, suggests that it is still important in natural infection. The EBNA3 proteins provide an important check on EBV-mediated viral and cellular transcription. EBNA3A, 3B and 3C contain a conserved region which is able to stably bind RBP-Jκ in a similar manner to EBNA2. Thus they compete with EBNA2 and Notch for RBP-Jκ and limit the strong EBNA2 up-regulation of viral and cellular promoters through RBP-Jκ (Johannsen et al., 1996; Marshall and Sample, 1995b; Robertson et al., 1996). The exact mechanism of repression is not fully understood; however EBNA3C has been shown to repress the viral Cp promoter activation by EBNA2 through binding to RBP-Jκ and by

association with histone deacetylase 1 (HDAC1) (Knight et al., 2003; Radkov et al., 1999). Aside from their regulatory properties, EBNA3 expression in B-lymphoma cells has been shown to exert modest changes in gene expression. In BJAB, EBNA3C up-regulates CD21, whereas EBNA3B in DG75 up-regulates vimentin, CD40 and Bcl-2 expression and down-regulates CD77 (Marshall and Sample, 1995a; Silins and Sculley, 1994).

There is increasing evidence implicating the EBNA3s in EBV-mediated oncogenicity and resistance to apoptosis. EBNA3C has been reported to cooperate with oncogenic Ras in the transformation of rat embryo fibroblasts (Parker et al., 1996). It also binds cyclin A and modulates cyclin A-dependent kinase activity in EBV-infected cells (Knight and Robertson, 2004; Knight et al., 2004).Transfection of the EBNA3s can rescue Latency I BL cells from apoptosis induced by serum withdrawal (Silins and Sculley, 1995). In NIH3T3 fibroblasts or human U2OS cells growth arrested by serum withdrawal, EBNA3C was also able to inhibit the accumulation of p27 (KIP1) and cause bi and multinucleated cells by abrogation of the mitotic spindle checkpoint (Parker et al., 2000). Accordingly, Wp restricted BL cell lines, where EBNA3s are expressed in the absence of EBNA2 and the LMPs, are significantly more resistant to apoptosis than conventional Latency I BL cells (Kelly et al., 2005). Additionally, EBV-negative BL cells could be rescued from apoptosis induced by cytotoxic drugs by expression of the EBNA3s from an EBNA2 knockout (KO) virus or transfection of EBNA3A and 3C, but not EBNA3B. This resistance to apoptosis was concomitant with a decrease in the expression of the pro-apoptotic Bcl-2 family member protein, Bim (Anderton et al., 2008; Leao et al., 2007). Thus, the EBNA3s may not only regulate viral transcription, but may also contribute to the oncogenicity and survival of EBV-infected cells.

1.8.5 LMP1

LMP1 is a 66 kDa integral membrane protein which is encoded by the BNLF1 ORF. The mature protein has a 24 aa arginine and proline rich hydrophilic N-terminal region, followed by 6 hydrophobic α helical transmembrane segments and a 200 aa C-terminus (Fennewald et al., 1984). The LMP1 protein is far less stable than the EBNAs, with a half life of only around 3-5 hours in LCLs (Hennessy et al., 1984). In LCLs, LMP1 is post-translationally inserted into the plasma membrane, where it forms discrete patches or larger caps (Liebowitz et al., 1986). Once inserted into the plasma membrane,

LMP1 acts as a constitutively active tumour necrosis factor receptor (TNFR), similar to CD40 or a combination of TNFRI and TNFRII (Izumi and Kieff, 1997; Mosialos et al., 1995).

LMP1 expression is fundamentally important for the transformation of B lymphocytes into LCLs by supporting LCL growth and survival. In LCLs which conditionally express LMP1, the loss of LMP1 expression halts the cell cycle and results in cell death within 5 days (Dirmeier et al., 2003; Kaye et al., 1993; Kilger et al., 1998). Interestingly however, these cells can survive quiescently if supported by fibroblast feeder layers (Dirmeier et al., 2005) indicating that LMP1 is not absolutely essential for LCL survival. When expressed independently of EBV infection, LMP1 increases the oncogenicity of several continuously proliferating cell lines. In Rat-1 or NIH 3T3 cells, LMP1 alters cell morphology, enables growth in medium containing low serum concentrations, causes a loss of contact inhibition and enhances tumourigenicity in nude mice (Wang et al., 1985). In a separate study in Rat-1 and Balb/c 3T3 cells, LCL-like levels of LMP1 protein again increased oncogenicity. However higher LMP1 levels were found to be toxic to the transfected cells (Martin and Sugden, 1991). Subsequently, LMP1 was also found to dramatically alter the growth of several human EBV-negative epithelial and B cell derived cell lines (Peng and Lundgren, 1992; Peng and Lundgren, 1993; Rowe et al., 1994; Wang et al., 1988). *In vivo*, expression of LMP1 in three lineages of transgenic C57/bl6 mice backcrossed into balb/c results in a marked increase in B cell hyperplasia and lymphomas (Kulwichit et al., 1998). Furthermore in humans, LMP1 expression can be found in both Hodgkin's Lymphoma (HL) and nasopharyngeal carcinoma (NPC).

In primary B cells, LMP1 induces many of the changes associated with EBV infection or antigen activation, including cell clumping, increased villous projections, increased surface expression of CD23, CD39, CD40, CD44, MHC class II, increased expression of adhesion molecules ICAM-1, LFA-1 and LFA-3, increased expression of IL-10 and decreased expression of CD10 (Wang et al., 1988). LMP1 expression is also able to protect B cells from apoptosis through an increase in Bcl-2 expression (Gregory et al., 1991; Henderson et al., 1991; Rowe et al., 1994).

Mutational analysis reveals that LMP1's cytoplasmic C-terminus and transmembrane domain 1 (TM1) are essential for B cell transformation (Kaye et al., 1993; Kaye et al., 1995). The cytoplasmic N-terminus is also important, but not critical for transformation, as its deletion results in a 90% reduction of transformation (Izumi et al., 1994). Further analysis has revealed that TM1 is essential for

aggregation of LMP1 proteins within the plasma membrane, while the cytoplasmic C terminus contains 2 critical transformation sites, referred to as C-terminus transformation effector sites (TES) 1 and 2 (Izumi et al., 1999a). TES1 and TES2 also correlate closely to the LMP1 carboxy-terminal NFκB activation domains, CTAR1 and CTAR2 (Huen et al., 1995; Mitchell and Sugden, 1995). TES1/CTAR1 binds to TRAF3 in the same manner as an activated CD40 pro-survival B cell receptor (Mosialos et al., 1995). TES2/CTAR2 interacts with TNFR-associated death domain (TRADD) protein and receptor interacting proteins (RIPs), but does not initiate a death signal (Izumi et al., 1999b). The interactions of TES1 and TES2 activate NFκB JNK, p38 and cdc42, while a third region positioned between TES1 and TES2, which is not essential for transformation, has been found to activate the JAK/STAT pathway via JAK3 binding (Eliopoulos and Young, 1998; Eliopoulos et al., 1999; Gires et al., 1999; Puls et al., 1999). In LCLs, LMP1-mediated activation of NFκB has a strong anti-apoptotic effect, since it has been shown that NFκB inactivation results in rapid apoptosis which can proceed even in the presence of high levels of Bcl-2 and BclXL (Cahir-McFarland et al., 2000; Cahir-McFarland et al., 2004). Thus LMP1 utilizes its position within the plasma membrane to influence large numbers of cellular genes and signalling pathways to promote the transformation of resting B cells into proliferating LCLs.

1.8.6 LMP2A/2B

In Latency III, EBV expresses 2 LMP2 proteins, LMP2A and LMP2B from unique EBNA2 responsive promoters containing multiple RBP-Jκ binding sites (Laux et al., 1988; Laux et al., 1994; Meitinger et al., 1994). LMP2A and LMP2B mRNAs share 8 common exons, but their first exons are unique. The first exon of LMP2A encodes a unique 119 aa N-terminal domain, which binds several signalling molecules. However in LMP2B this exon is non-coding and translation begins in exon 2. Both proteins encode 12 transmembrane (TM) domains separated by short reverse turns and a 27 aa C-terminal cytosolic domain (Laux et al., 1989; Sample et al., 1989). In LCLs, the hydrophobic TM domains mediate LMP2A self association and colocalization with LMP1 to lipid rafts within the cell membrane (Higuchi et al., 2001; Longnecker and Kieff, 1990).

Neither LMP2A nor LMP2B appears to be essential for EBV's transformation of primary B cells. Analysis of a number of EBV recombinants suggested that loss of the LMP2 gene from EBV does not

affect transformation efficiency and that the resultant LCLs display the same growth and virus replication characteristics as cells infected with WT EBV (Kim and Yates, 1993; Longnecker et al., 1992; Longnecker et al., 1993a; Longnecker et al., 1993b; Speck et al., 1999). However, a number of apparently conflicting studies found that loss of the LMP2 gene reduced transformation efficiency or restricted transformation to B cells with a functional B cell receptor (BCR) (Brielmeier et al., 1996; Mancao and Hammerschmidt, 2007). While it may not be critical for transformation, LMP2A has been shown to provide a powerful survival and anti-differentiation signal to B cells. LMP2A expression in BL and gastric carcinoma cell lines was able to protect cells from BCR cross-linking and transforming growth factor beta (TGFβ) associated apoptosis (Fukuda and Longnecker, 2004; Fukuda and Longnecker, 2005) and in LCLs, LMP2A also cooperates with LMP1 to enhance B cell survival by increasing NFκB signalling (Dawson et al., 2001; Guasparri et al., 2008). In transgenic mice, LMP2A under the control of the Ig heavy chain promoter and enhancer appears to act as a surrogate light chain on the surface of pre-B cells. LMP2A expression enabled surface Ig negative B cells to escape from the bone marrow and colonize peripheral lymphoid organs by bypassing the normal B lymphocyte checkpoints (Caldwell et al., 1998; Caldwell et al., 2000). LMP2A expression during B cell development in mice also decreased expression of B cell development factors, E2A, EBF and Pax5 (Portis et al., 2003). As discussed in section 1.13.2 LMP2A signalling is also important for the pathogenesis of Latency II HL and NPC; thus LMP2A can mediate sufficient constitutive forward signalling to affect normal B cell survival.

As well as providing survival and anti-differentiation signals, the unique N-terminal domain of LMP2A is able to interact with the B lymphocyte Src family tyrosine kinases, especially Lyn (Pleiman et al., 1994) through its immunoreceptor tyrosine-based activation motif (ITAM) (Merchant et al., 2000). Binding of Lyn results in the phosphorylation of all the LMP2A tyrosine residues and leads to the recruitment of Syk and the activation of PI3K, Btk, BLNK and Akt (Merchant and Longnecker, 2001; Swart et al., 2000). Mutagenic analysis of LMP2A shows that signal transduction by Lyn and Syk through the N-terminal domain of LMP2A constitutively blocks the activation of the EBV lytic cycle that would normally follow cross linking of CD19, MHC class II or the B cell receptor (BCR) (Miller et al., 1993; Miller et al., 1994a). Thus a second important role of LMP2A may be to inhibit lytic replication in latently infected B cells.

By contrast, the function of LMP2B is still largely unknown; however it is believed to antagonise the action of LMP2A. LMP2B has been shown to colocalize with LMP2A on the plasma membrane and disrupt its self association (Lynch et al., 2002). Coexpression of LMP2B with LMP2A also restored normal BCR signal transduction upon BCR cross-linking, thus alleviating the LMP2A-mediated block on the activation of the EBV lytic cycle (Rechsteiner et al., 2008; Rovedo and Longnecker, 2007).

1.8.7 EBERs

The two non-polyadenylated, non-coding EBER transcripts are expressed in all forms of viral latency (Alfieri et al., 1991). The 166 nucleotide EBER1 and 172 nucleotide EBER2 transcripts are the most abundantly expressed EBV RNA transcripts in latently infected cells, with an estimated 10^7 copies per cell (Arrand and Rymo, 1982; Howe and Steitz, 1986). In LCLs, EBER1 is about ten-fold more abundantly expressed than EBER2 (Howe and Shu, 1989; Howe and Shu, 1993). Once transcribed, the EBERs form complex secondary structures and assemble into stable ribonucleoprotein particles with the autoantigen, La (Lerner et al., 1981) and with ribosomal protein L22 (Toczyski et al., 1994). The 3' terminus of both EBER transcripts associates with the La protein, while three sites within EBER1 also associate with L22 (Fok et al., 2006; Glickman et al., 1988). It appears that most EBER1 is bound to L22, as an anti L22 antibody will precipitate nearly all EBER1 transcripts. However, despite the fact that L22 shuttles from the nucleus to the cytoplasm the vast majority of EBER transcripts remain within the nucleus (Schwemmle et al., 1992).

There is currently some disagreement over the requirement of EBERs for transformation of B cells *in vitro*. Initially, a recombinant virus lacking EBERs was found to be equivalent to WT virus in its efficiency of B cell transformation, LCL morphology, EBV latent and early lytic gene expression and LCL regrowth after dilution in fresh medium (Swaminathan et al., 1991). However, more recent studies found that loss of EBER2 but not EBER1 significantly impaired the ability of EBV to transform B lymphocytes (Wu et al., 2007; Yajima et al., 2005). In transformation assays, deletion of both EBERs or EBER2 alone increased 100-fold the dose of virus required to transform 50% of cord blood B lymphocyte cultures. Growth of the resultant EBER KO LCLs was slow especially at low densities but could be enhanced by supplementation with IL-6, indicating an EBER-mediated role for this interleukin in transformation. Restoration of EBERs to the KO virus increased transformation efficiency and IL-6

production and restored growth capacity to the level of the neo control virus. Discrepancies between these studies may result from the use of irradiated LCLs as a source of infection in the first study, which may have compensated for the loss of EBERs.

Despite the apparent requirement for EBERs for transformation of B cells *in vitro*, their function has yet to be fully elucidated. EBERs share similar primary sequence and secondary structures to the adenovirus VA1 RNA (Arrand et al., 1983), which blocks interferon (IFN)-induced RNA activated protein kinase R (PKR) phosphorylation and the subsequent inhibition of the translational initiation factor eIF-2α (Kitajewski et al., 1986). EBER1 and EBER2 can functionally substitute for VA1 during late lytic replication of adenovirus 5 and can inhibit PKR with similar efficiencies to VA1 *in vitro* (Bhat and Thimmappaya, 1985). EBERs bind to PKR in a cell free system and as PKR has a role in mediating the antiviral effects of the interferons, it has been suggested that EBER-mediated inhibition of PKR function could be important for viral persistence (Clemens et al., 1994).

As discussed in more detail in section 1.15.2, EBERs have also been implicated in the tumourgenicity of BL cells. Spontaneous loss of EBV from BL cells has been shown to increase susceptibility to apoptosis, reduce ability of cells to grow in soft agar and form tumours in severe combined immunodeficient (SCID) mice and decrease Bcl-2 expression; all of which could be reversed by reinfection with EBV or expression of the EBERs (Shimizu et al., 1994; Komano et al., 1998; Komano and Takada, 2001). It is proposed that EBERs mediate this effect through modulation of the IFN response via binding to PKR (Komano et al., 1999; Nanbo et al., 2002; Nanbo et al., 2005; Ruf et al., 2000; Sharp et al., 1993) and by induction of the autocrine growth factor IL-10 (Kitagawa et al., 2000; Samanta et al., 2008). Thus, despite their non coding nature, the ubiquitously expressed and highly stable EBERs may play an important role in normal EBV infection and in EBV-mediated pathogenesis.

1.8.8 Other EBV latent transcripts

1.8.8.1 *Bam*HI-A rightward transcripts (BARTs)

*Bam*HI-A rightward transcript (BART) mRNAs were initially discovered in NPC samples (Hitt et al., 1989; Gilligan et al., 1990), but have been subsequently discovered at low levels in all forms of latent and lytic EBV infection, both *in vitro* and *in vivo* (Bell et al., 2006; Brooks et al., 1993; Chen et al., 1992a; Karran et al., 1992; Sadler and Raab-Traub, 1995b; Zhang et al., 2001). Numerous transcripts

are generated from the BART region including the mRNAs which encode the RK-BARF0 (Fries et al., 1997; Kienzle et al., 1998; Kusano and Raab-Traub, 2001), A73 (Smith et al., 2000) and RPMS1 proteins (Smith et al., 2000). The introns from this region also encode a number of microRNAs (miRNA), which are discussed further in section 1.8.8.2. Transcription is believed to originate from two TATA-less promoter regions, P1 and P2, at B95-8 EBV coordinates 150641 and 150357 (Baer et al., 1984; de Jesus et al., 2003). P1 is the initial B cell promoter, but is also active in epithelial cells and is down regulated by interferon regulatory factor (IRF) -7 and -5. P2 is more active in epithelial cells and is up-regulated by c-Myc and C/EBP (Chen et al., 2005).

Despite the ubiquitous expression of BART mRNAs, their function remains elusive, as deletion of the BART coding region in EBV strains such as the commonly used B95-8 virus does not appear to significantly impair its transformation of B lymphocytes. Nevertheless as described in section 1.15.3, potential BART derived proteins have been implicated in modulation of cellular pathways including the highly conserved notch signalling cascade (Kusano and Raab-Traub, 2001; Thornburg et al., 2004; Smith et al., 2000). However, despite these potentially interesting interactions, RK-BARF0, A73 and RPMS1 protein has not been detected in EBV-infected human cells and humans have little detectable antibody to these proteins (van Beek et al., 2003).

1.8.8.2 EBV encoded microRNAs

MicroRNAs (miRs) are single stranded 21-23 nucleotide RNA molecules which modulate gene expression. After processing from pre-miRs they bind partially complementary mRNA sequences resulting in degradation of the mRNA transcripts and a reduction in gene expression. EBV is currently believed to encode 42 miRs, although new miRs continue to be discovered as detection techniques improve (Barth et al., 2008; Cosmopoulos et al., 2008; Cai et al., 2006; Grundhoff et al., 2006; Pfeffer et al., 2004; Zhu et al., 2009). Figure 1.5 is adapted from Barth et al., 2008 and shows the location of 23 most well characterised of the EBV encoded miRNAs, which are clustered in 3 regions within the EBV genome.

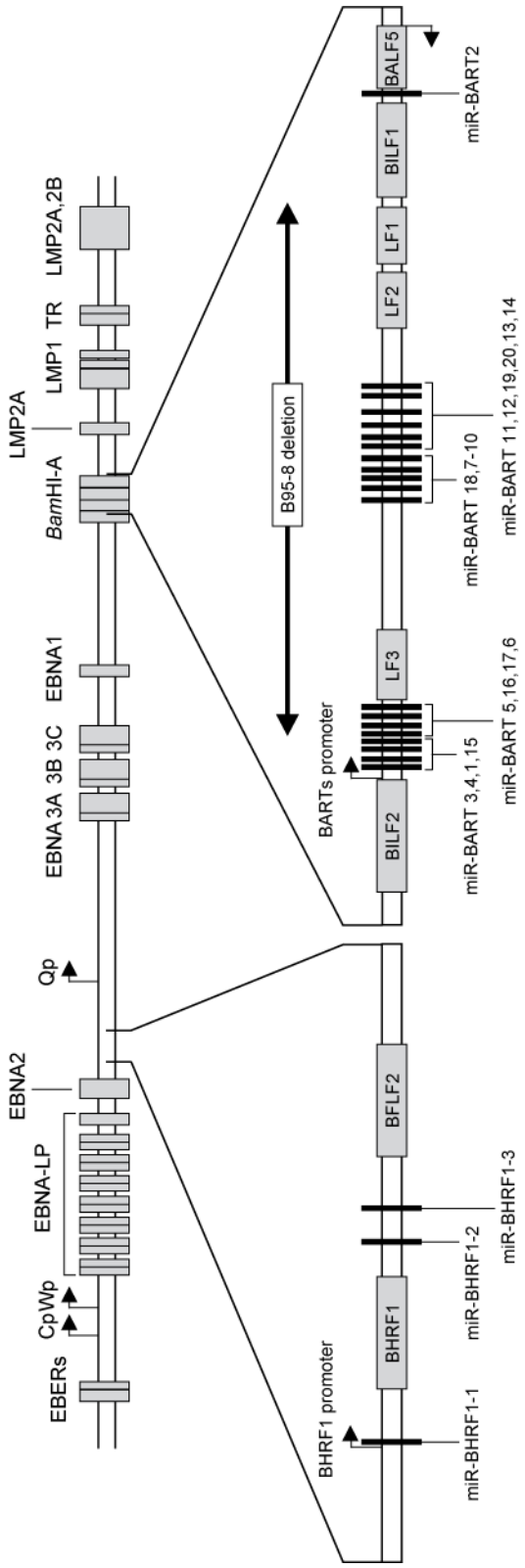


Figure 1.5. This figure is adapted from Barth et al., 2008 and shows the location of virally encoded miRNAs within the EBV genome. The top row show the position of genes expressed in Latency III and the bottom row shows in more detail the position of the three clusters of miRNAs and the location of the B95-8 deletion. Grey boxes indicate exons and the terminal repeats (TR), while the black lines indicate the position of the miRNAs.

The three BHRF1 miRs (miR-BHRF1-1, -2 and -3) flank the BHRF1 ORF, while the remaining 20 miRs are transcribed from 2 regions within the introns of the BART region. miR-BART3, 4, 1, 15, 5, 16, 17 and 6 lie between the BILF2 and LF3 exons adjacent to the BARTs promoter, while miR-BART8, 7-12, 19, 20, 13 and 14 are located between LF3 and LF2. As shown in Figure 1.5, the prototypic B95-8 strain has a deletion which results in loss of all but 7 of these miRNAs (miR-BHRF1-1, -2, -3, miR-BART1, 2, 3 and 4).

The expression patterns of the various EBV miRs are fairly complex and appear to depend on both the cell type and on the overall pattern of EBV gene expression. The BART miRs are robustly expressed in Latency II NPC cell lines, but have also been detected at a lower abundance in other EBV-infected B cell lines. The BHRF1 miRNAs are likely to be derived from an intron generated by splicing of the Cp and Wp initiated transcripts, hence are found exclusively in B cells with a Latency III infection (Cai et al., 2006; Xing and Kieff, 2007). Additionally, activation of EBV lytic replication induces expression of many, but not all, of the virally encoded miRs.

Loss of the majority of miRs in B95-8 does not ablate transformation of B lymphocytes; however there is a high degree of evolutionary conservation between the miRs of EBV and those of the rhesus lymphocryptovirus, indicating that they play an important role in some stage of the EBV life cycle (Cai et al., 2006; Xing and Kieff, 2007). In agreement with this, both cellular and viral targets have been discovered for the EBV encoded miRs. The cellular IFN-inducible T-cell attracting chemokine, CXCL-11/I-TAC and the pro-apoptotic Bcl-2 family member protein, p53 up-regulated modulator of apoptosis (Puma) have been identified as targets of miR-BHRF1-3 and miR-BART5 respectively, eluding to a possible role for EBV-encoded miRNAs in EBV-mediated proliferation and apoptosis resistance (Xia et al., 2008; Choy et al., 2008).

Two viral genes also appear to be modulated by EBV encoded miRs. The first of these is the EBV lytic cycle DNA polymerase gene, BALF5, which is transcribed from the opposite strand as the one encoding miR-BART2. In reporter assays, miR-BART2 expression decreased BALF5 expression and, in lytically active cells, miR-BART2 overexpression reduced the release of infectious EBV particles indicating a potential role in the inhibition of lytic replication in latently infected cells (Barth et al., 2008). A second EBV gene whose expression appears to be modulated by EBV miRs is LMP1. While physiological LMP1 expression leads to NFκB activation, overexpression of LMP1 reduces NFκB

activation, inhibits proliferation and increases susceptibility to apoptosis (Eliopoulos et al., 1996; Kaykas and Sugden, 2000). Several partly complementary matches to BART miRs have been found in the 3' UTR of LMP1 (Lo et al., 2007) and miR-BART expression was found to suppress LMP1 protein production, protect against apoptotic stimuli and prevent NFκB inhibition. Overall, understanding of EBV encoded miRs is still in its infancy, but it is becoming increasingly apparent that they modulate expression of a broad range of cellular and viral genes.

1.8.8.3 BHRF1 and BALF1

The BHRF1 gene is found adjacent to the EBV origin of lytic replication and is expressed during the EBV early lytic cycle. BHRF1 protein is highly anti-apoptotic; it shares homology with the anti-apoptotic Bcl-2 protein but, unlike Bcl-2, does not bind Bax, Bak, Bid or Bad (Huang et al., 2003). It is believed that expression of BHRF1 maintains the viability of cells entering lytic cycle, therefore extending the time over which viral progeny can be produced (Henderson et al., 1993; Sample and Kieff, 1990).

The BALF1 gene is located close to the LMP1 ORF. Whilst it is also a Bcl-2 homologue expressed during the early lytic cycle, its role in apoptosis resistance remains controversial. It clearly has anti-apoptotic properties (Cabras et al., 2005; Marshall et al., 1999), but it has also been shown to antagonise the anti-apoptotic effects of BHRF1 (Bellows et al., 2002).

Although BHRF1 and BALF1 are primarily lytic cycle antigens, the BHRF1 coding sequence can also be found in Latency III infected cells at the 3' end of some Cp/Wp-initiated EBNA transcripts (Austin et al., 1988; Sample and Kieff, 1990). BHRF1 protein has recently been detected in Wp restricted BL cells and long established LCLs (Kelly et al., 2009 (in press)). Recombinant EBV with a nonsense mutation or deletion of the BHRF1 ORF transforms primary B lymphocytes with efficiencies similar to WT virus (Lee and Yates, 1992; Marchini et al., 1991); however transformation ability is abolished upon genetic inactivation of both BHRF1 and BALF1 (Altmann and Hammerschmidt, 2005). This indicates that a degree of redundancy exists between these two proteins, but that the anti-apoptotic signals that they provide are essential for transformation.

1.9 EBV lytic cycle

The EBV lytic cycle can be subdivided into the immediate early (IE), early and late lytic cycle by the expression of distinct lytic genes. The IE genes are expressed immediately following lytic cycle induction and transcription occurs even in the presence of inhibitors of protein synthesis, such as cycloheximide (Biggin et al., 1987; Flemington et al., 1991; Takada and Ono, 1989). Once activated, the IE gene products function as transcription factors which activate expression of the early lytic genes. Early lytic genes are defined as those which are transcribed prior to viral DNA replication; thus they are expressed in the presence of inhibitors of viral DNA replication, such as acyclovir (ACV), but their expression is inhibited by treatment with inhibitors of protein synthesis. Finally, the late lytic genes are expressed; these include several structural virion proteins and they are defined by their inhibition by inhibitors of viral DNA synthesis.

1.9.1 Immediate early lytic cycle

In the human host, it is likely that activation of the EBV lytic cycle is initiated by differentiation of infected B cells into plasma cells following antigen stimulation of the BCR (Laichalk and Thorley-Lawson, 2005) or by differentiation of infected squamous epithelial cells (Tovey et al., 1978; Young et al., 1991). There is also evidence to suggest that lytic reactivation could be stimulated by certain cytokines, particularly TGF-beta (Fahmi et al., 2000), by interaction between EBV-infected B cells and CD4 T lymphocytes or in response to toxic stimuli including chemotherapy and irradiation (Feng et al., 2002). In cell culture systems *in vitro*, infected cells can be artificially induced into viral replication by surface Ig cross linking, treatment with phorbol ester, 12-0-tetradecanoyl phorbol-13-acetate (TPA), sodium butyrate or a calcium ionophore (Angel et al., 1987; Flemington and Speck, 1990; Takada and Ono, 1989). Of all the methods used to induce the EBV lytic cycle, the most extensively studied is the engagement of the B cell receptor, which in the Akata-BL cell line can synchronously induce up to 50% of cells into lytic replication. In Akata-BL cells, viral IE gene expression occurs very rapidly (30 minutes or less) after B cell receptor engagement (Takada and Ono, 1989; Mellinghoff et al., 1991; Flemington et al., 1991) and requires activation of phosphatidylinositol 3-kinase (PI3K) and calcium-

dependent signalling pathways (Adamson et al., 2000; Chatila et al., 1997; Darr et al., 2001; Iwakiri and Takada, 2004).

All of the lytic cycle initiation treatments ultimately share an ability to activate transcription of the IE genes, BZLF1 and BRLF1 and in some cell lines the EBV lytic cycle can also be initiated by direct expression of BZLF1 (Flemington and Speck, 1990; Johannsen et al., 2004). The BZLF1 and BRLF1 proteins function as transcription factors, which activate their own promoters (Zp and Rp), as well as one another's promoters and thus greatly amplify the inducing effect of the initial lytic stimulus (Liu and Speck, 2003; Adamson et al., 2000; Flemington et al., 1991; Ragoczy and Miller, 2001; Sinclair et al., 1991; Speck et al., 1997; Zalani et al., 1996). The BZLF1 protein is a viral homologue of c-Jun and c-Fos, which activates early EBV lytic infection via binding to AP-1 like domains known as Z-response elements (ZRE) (Chang et al., 1990; Packham et al., 1990). BZLF1 mRNA has 3 domains, each of which encodes a separate functional domain (Farrell et al., 1989; Lieberman and Berk, 1990). The first exon encodes the N-terminal transactivation domain (aa 1 to 167), which is essential for replication but not transcriptional activation (Sarisky et al., 1996); the second exon (aa 168 to 202) includes a strongly basic domain with homology to a conserved region of the c-Fos and c-Jun family of transcriptional regulators (Mikaelian et al., 1993); while the third exon (aa 203 to 245) includes a leucine and isoleucine heptad repeat which enables coiled-coil interaction and facilitates BZLF1 homodimerisation (Carey et al., 1992). BRLF1 is also a DNA sequence specific acidic transactivator; it has distant homology to c-myb, which can interact synergistically with BZLF1 in transactivation of the early lytic BMLF1 promoter (Kenney et al., 1992). BRLF1 aa 416 to 519 are weakly activating in B lymphocytes, whereas the carboxy-terminal aa 520 to 650 are a potent acidic activator (Hardwick et al., 1992).

1.9.2 Early lytic cycle

Inhibition of viral DNA synthesis after cross linking of surface Ig in Akata-BL cells identified 38 EBV early lytic mRNAs (Lu et al., 2006; Yuan et al., 2006), including the EBV DNA polymerase (BALF5) and the major DNA binding protein (BALF2). Expression of early lytic viral proteins results in the replication of EBV genomes as very large linear concatemeric EBV DNA structures through rolling

circle replication (Cho and Tran, 1993). The EBV early lytic genes are intermingled throughout most of the EBV genome; some early lytic genes are spliced, while others are not.

The BMLF1 gene product, SM, is the most rapidly induced early lytic protein following Ig cross-linking in Akata-BL cells (Takada et al., 1991; Yuan et al., 2006) and is essential for EBV lytic replication (Gruffat et al., 2002; Ruvolo et al., 2004). The role of SM is to increase efficiency of EBV encoded RNA processing. SM increases the export of intronless viral RNA into the cytoplasm and increases the 3' processing efficiency and stability of the EBV polymerase, which is intrinsically deficient in 3' processing (Furnari et al., 1992). Amino acids 60-140 and 218-237 are nuclear export signals (Boyle et al., 1999; Chen et al., 2001), aa 152-172 form an RNA binding domain (Buisson et al., 1999) and residues 173-203 and 470- 474 are core hydrophobic residues essential for SM function (Ruvolo et al., 2004).

During the early lytic cycle, EBV also expresses BHRF1 and BALF1, which are structural homologues of the anti-apoptotic Bcl-2 protein (Bellows et al., 2002; Henderson et al., 1993; Pearson et al., 1983; Marshall et al., 1999). BHRF1 has been shown to suppress apoptosis in lymphoid cells in response to a range of triggers including serum deprivation (Henderson et al., 1993), DNA damaging agents, chemotherapeutic drugs (McCarthy et al., 1996) and cytokines (Foghsgaard and Jaattela, 1997). Although BHRF1 is dispensable for EBV replication (Lee and Yates, 1992; Marchini et al., 1991), it is very highly conserved in distinct geographical isolates of EBV (Khanim et al., 1997) and is believed to maintain the viability of cells entering lytic cycle *in vivo* (Henderson et al., 1993; Sample and Kieff, 1990). The role of the BALF1 protein is less well characterised. When expressed in HeLa cells, BALF1 associates with the pro-apoptotic Bcl-2 proteins, Bax and Bak and increases resistance to apoptosis (Marshall et al., 1999), while in BL cells BALF1 enabled growth in low serum (Cabras et al., 2005). However, BALF1 was also found to antagonise the anti-apoptotic activity of BHRF1 as well as the KSHV Bcl-2 homologue, KSBcl-2 (Bellows et al., 2002). The anti-apoptotic role of these proteins appears to be essential for the early stages of B cell transformation as genetic inactivation of both BHRF1 and BALF1 inhibits LCL formation (Altmann and Hammerschmidt, 2005).

Many of the EBV lytic cycle antigens are highly immunogenic. To reduce the susceptibility of infected cells to CTL recognition during the reactivation of the lytic cycle *in vivo*, EBV expresses BNLF2a. The BNLF2a protein blocks both the ATP and peptide binding capacity of the transporter associated with

antigen processing (TAP), which in turn reduces expression of surface human histocompatibility leukocyte antigen (HLA) class I (Hislop et al., 2007).

1.9.3 Late lytic cycle

Once EBV DNA has been replicated, lytically active cells begin to express late lytic antigens. In Akata-BL cells induced into lytic cycle by engagement of the B cell receptor, 40 EBV late lytic mRNAs have been identified (Lu et al., 2006; Yuan et al., 2006). As observed for the early lytic genes, some of these genes are spliced while others are not. 28 of the late lytic mRNAs are translated into virion proteins, including the EBV glycoproteins, the capsid proteins, the tegument proteins and the virion kinase. Once the virion proteins are expressed, the viral protease BVRF2 provides a scaffold for the assembly of virion proteins into viral particles ready for the encapsulation of viral DNA (Donaghy and Jupp, 1995).

Non-structural late lytic cycle proteins include BCRF1, which shares nearly 90% colinear aa sequence with human IL-10 (Hsu et al., 1990; Vieira et al., 1991). Like human IL-10, BCRF1 or viral IL-10 (vIL-10) is an autocrine growth factor capable of paracrine signalling to surrounding B cells. vIL-10 signalling enhances cell survival and proliferation, modulates expression of the NFκB and the JAK-STAT signalling pathways and increases expression of the Th1 cytokines (Ding et al., 2000; Ding et al., 2001; Stuart et al., 1995). However, unlike cellular IL-10, vIL-10 lacks the ability to sustain murine mast cell lines or modulate MHC class II expression. vIL-10 is not critical for the outgrowth of LCLs and recombinant viruses with BCRF1 nonsense mutations or deletions transform B lymphocytes with equal efficiency to WT viruses (Swaminathan et al., 1993). It is possible therefore that the major role of vIL-10 may be one of immune suppression. Accordingly vIL-10 has been shown to be a negative regulator of macrophage and NK cell function, which would ordinarily mediate release of IFN-γ from cytotoxic T lymphocytes upon contact with an infected cell (Moore et al., 1993). *In vitro*, incubation of peripheral blood mononuclear cells (PBMCs) with WT LCLs results in low IFN-γ release; however incubation with BCRF1 null mutants results in high IFN-γ and inefficient transformation of B lymphocytes. Thus *in vivo*, vIL-10 may function by blunting the NK and T cell response to EBV infection.

In Akata-BL cells induced into lytic cycle by engagement of the B cell receptor, completion of the lytic cycle takes between 48-72 hours and culminates with the deterioration of the cell and the release of large quantities of enveloped virus particles (Takada and Ono, 1989).

1.9.4 Genes expressed in both latent and lytic EBV infection

Even after the switch from latent to lytic EBV gene expression, some latency-associated proteins continue to be expressed. EBNA1 is expressed in lytic cycle, but transcription now initiates from the lytic Fp promoter, located in the *Bam*HI-F fragment (Lear et al., 1992; Schaefer et al., 1995a) and in some systems LMP1 promoters remain active in the early lytic cycle (Rowe et al., 1992). Interestingly however, expression of LMP1 during the lytic cycle results in the expression of a truncated form of the LMP1 protein (Hudson et al., 1985), while the other EBNAs and LMPs appear to be down-regulated, possibly due to repression of Cp by BZLF1 (Kenney et al., 1989). Also, as previously noted, an anti-apoptotic signal from BHRF1 and or BALF1 appears to be essential to establish a latent infection in B cells (Altmann and Hammerschmidt, 2005).

1.10 B cell development and immunoglobulin maturation

EBV is strongly B lymphotrophic and highly transforming *in vitro* (Henle et al., 1967). *In vivo* EBV normally persists asymptomatically as a lifelong infection (Miyashita et al., 1995; Miyashita et al., 1997), but is also associated with several B cell lymphomas (Epstein et al., 1964a; Locker and Nalesnik, 1989; Magrath, 1990; Weiss et al., 1987; Weiss and Movahed, 1989; Zutter et al., 1988). Consequently, to fully understand EBV infection and persistence *in vivo* as well as the role of EBV in B cell lymphomagenesis, it is important to gain an understanding of normal B cell development.

1.10.1 The immunoglobulin receptor

B cells recognise and respond to antigens through the unique binding properties of their immunoglobulin (Ig) receptor molecules. As shown in Figure 1.6, Ig receptors are Y-shaped molecules composed of two identical light (L) chains and two identical heavy (H) chains, held together by a combination of noncovalent and covalent (disulphide) bonds. There are five classes of heavy chain α, δ, ε, γ and μ; these determine the Ig class, IgA, IgD, IgE, IgG and IgM, respectively. Each class of Ig

can also have either κ or λ light chains, which appear to be functionally indistinguishable. Together the light and heavy chains form a unique antigen binding site at the tip of each arm.

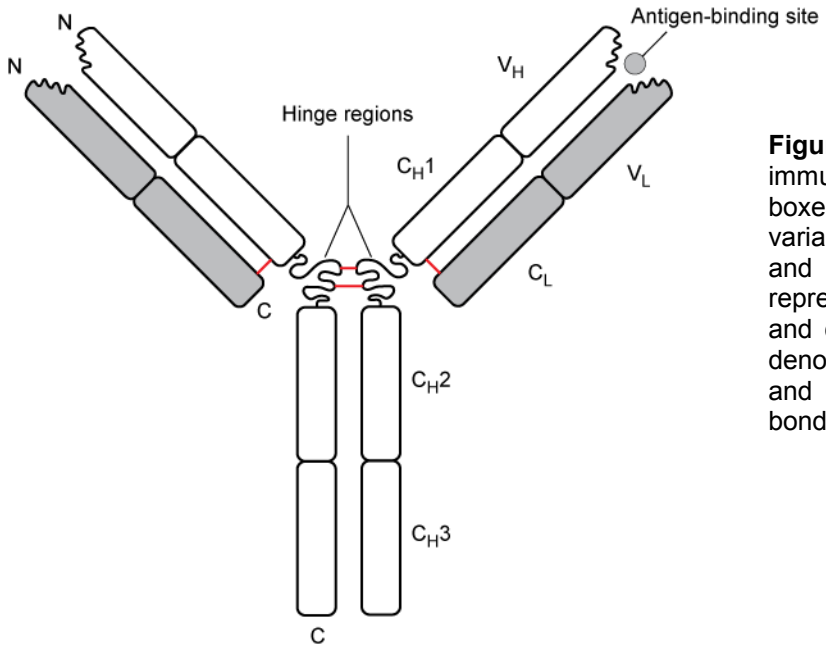


Figure 1.6. Schematic diagram of the immunoglobulin (Ig) receptor. White boxes represent the heavy chain variable (V_H) and constant (C_H1, C_H2 and C_H3) domains, grey boxes represent the light chain variable (V_L) and constant (C_L) domains, N and C denote the amino and carboxy termini and red lines represent disulphide bonds.

Comparison of the aa sequences of different antibody molecules reveals that both light and heavy chains have a variable (V) region at their N-terminal ends, which is responsible for antigen specificity and a constant (C) region at their C-terminal ends, which determines functional activity of the Ig molecule and specifies isotype. The C region of each Ig chain is encoded by a single region of DNA, whereas the variable regions are composed of multiple gene segments. The light chain V region is encoded by two gene segments, a long variable ($V_{L)}$ gene segment and a short joining (J_L) gene segment, while the heavy chain variable region is encoded by V_H and J_H segments plus an additional diversity (D_H) gene segment (Tonegawa, 1983). The large numbers of inherited V, J and D gene segments available for encoding Ig chains makes a substantial contribution to Ig diversity. For example, any of the 40 V segments in the human κ light-chain gene segment pool can be joined to any of the 5 J segments and, similarly, any of the 51 V segments in the heavy chain pool can be joined to any of the 6 J segments and any of the 27 D segments.

1.10.2 V(D)J recombination

The process by which V, D and J regions are assembled into a functional Ig receptor is shown in Figure 1.7 and is known as V(D)J recombination (Market and Papavasiliou, 2003). This process is mediated by an enzyme complex called V(D)J recombinase, which contains the products of the lymphocyte specific, recombination activating genes (RAG) 1 and 2, as well ubiquitous DNA repair proteins. The RAG proteins introduce double-strand breaks at sequences flanking the light and heavy chain gene segments. This is then followed by a rejoining process that is mediated by both the RAG proteins and the general DNA double-strand repair proteins. During the joining of the Ig gene segments, a variable number of nucleotides are lost from or inserted on to the ends of the recombining gene segments. This random loss and gain of nucleotides at the joining sites is called junctional diversification. The change in nucleotide length frequently results in a frame shift in the Ig gene rendering it non-functional and halting B cell development. However in cells able to express a functional Ig this process increases the diversity of V-region coding sequences created by recombination by more than 10^8 fold.

1.10.3 B cell development

As shown in Figure 1.8, the stage of B cell development is defined by the various stages of maturation of the Ig receptor (Kokron et al., 2004). In humans, B cells originate from haematopoietic stem cells within the bone marrow. These lymphoid progenitor cells receive signals from bone marrow stromal cells to begin B cell development as early pro-B cells. Cytokines induce RAG-1 and RAG-2 and the early pro B-cells undergo D-J joining of the Ig_H chain loci. Joining of a V segment to the D-J_H takes place in late pro-B cells and results in the formation of an intact μ heavy chain, which is expressed transiently with a surrogate light chain to form the pre B-cell receptor on the surface of large pre-B cells. Expression of an intact pre-B cell receptor halts heavy chain rearrangement, down-regulates expression of the RAG genes and stimulates cells to rapidly proliferate into small pre-B cells (Grawunder et al., 1995). Those cells unable to generate an intact pre-B cell receptor due to frame shifting during V(D)J recombination die by apoptosis. Light chain rearrangement then occurs in surviving cells. Initially rearrangement commences at the two κ loci but, if this is unsuccessful, rearrangement switches to the λ loci (Hieter et al., 1981; Engel et al., 1999). Successful

rearrangement of light chain genes then down-regulates expression of the pre-B cell receptor and a complete IgM molecule is expressed on the cell surface, at which point the cell is defined as an immature B cell.

Newly formed immature B cells are then negatively selected to remove cells whose receptors bind self-antigen in the bone marrow (Nemazee et al., 1991). Self-reactive B cells are stimulated to re-express the RAG proteins and undergo a second round of V(D)J rearrangements, thereby changing the specificity of their cell surface Ig; a process referred to as receptor editing (Nemazee and Weigert, 2000). B cells that fail to change their specificity after this second round of Ig rearrangement are eliminated through the process of clonal deletion, while the remaining positively selected Ig-expressing B cells exit the bone marrow as mature, naïve (antigen inexperienced) B cells. After leaving the bone marrow, circulating naïve B cells produce cell surface IgD molecules with the same antigen binding site as their existing IgM molecules and are now fully equipped to respond to foreign antigen.

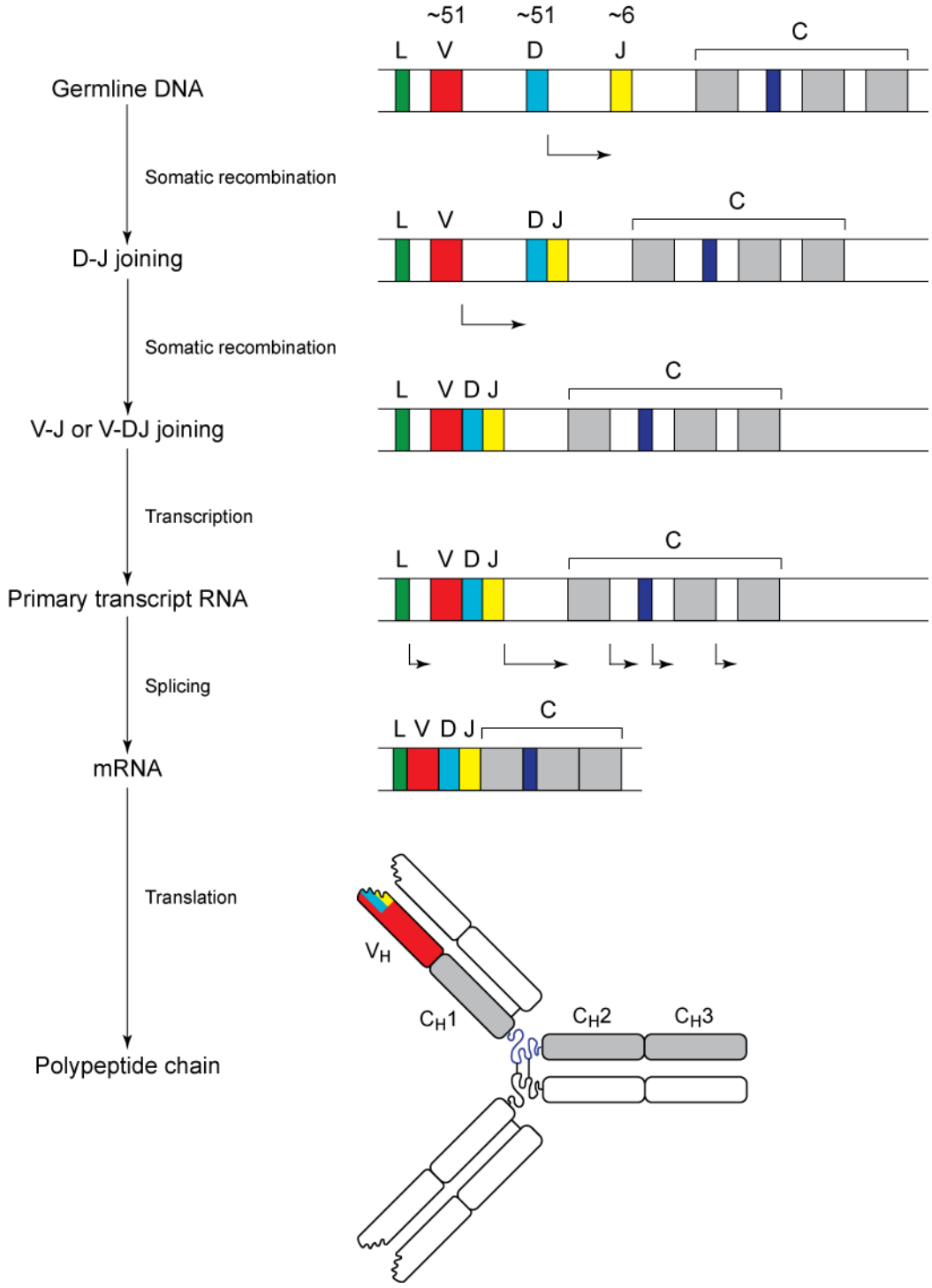


Figure 1.7. This figure is adapted from Market and Papavasiliou, 2003 and shows a schematic representation of the sequential rearrangement of the Ig heavy chain to form a functional polypeptide chain by V(D)J recombination. A single diversity (D) region joins a joining (J) region. The heavy chain variable region is then completed upon the addition of a variable (V) region and during transcription the VDJ_H region is spiced on to the leader (L) and constant (C) regions. The heavy chain is then translated and incorporated into a functional immunoglobulin molecule. Multiple V, D and J regions exist, although only one is shown. Light chain rearrangement occurs in the same way although, because of the absence of a D region, the V gene segment joins directly to the J segment.

1.10.4 Affinity maturation

Binding of the BCR on circulating naïve B cells to the appropriate antigen causes B cell aggregation and low level activation. The antigen is then internalised, processed in the endosomal pathway and antigenic peptides presented back on the cell surface, complexed to MHC class II molecules for recognition by helper T cells (Lanzavecchia, 1990). Once engaged, these antigen-specific helper T cells deliver further activating signals to B cells in the form of CD40 ligation and cytokine release (Valle et al., 1989), which induces formation of a primary focus of clonal expansion where proliferation of both B and T lymphocytes occurs. After several days of proliferation, the B cells differentiate into antibody secreting plasma cells or migrate with a small proportion of T cells to the primary lymphoid follicle and form a germinal centre (GC). Cells residing within the GC divide every 6-8 hours and during each cell cycle somatic hypermutation (SHM) of the V region introduces a 1bp point mutation in every 10^3 base pairs (Berek, 1992; Kelsoe, 1995; Kosco and Gray, 1992; MacLennan, 1994). Most mutations result in reduction of Ig avidity for antigen or a complete ablation of BCR expression resulting in B cell death by apoptosis. However, a small minority of the altered antigen receptors generated by hypermutation have increased affinity for the antigen and are preferentially stimulated to survive and proliferate in a process termed affinity maturation. Finally, class switching and further differentiation within the selected clones takes place to generate highly specific antibody secreting plasma cells or resting memory B cells (Liu et al., 1996). EBV has been associated with tumours from a wide range of different B cell developmental stages and the presence of Ig hypermutations can be diagnostic of the origin of the tumour progenitor cell.

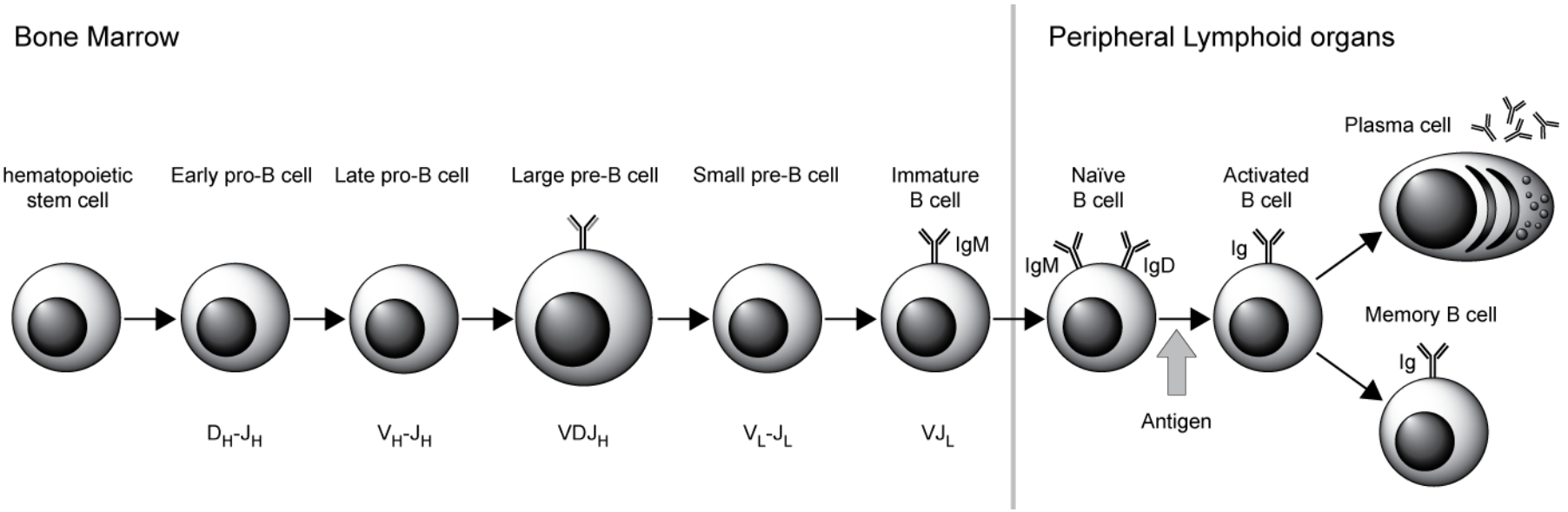


Figure 1.8. This figure is adapted from Kokron et al., 2004 and shows a schematic representation of B cell maturation and the accompanying immunoglobulin expression and rearrangement. Heavy chain rearrangement occurs first and an intact μ chain is expressed on the surface of the Large pre-B cell along with a surrogate light chain (grey line). Differentiation to an immature B cell is then accompanied by light chain rearrangement and expression of an IgM B cell receptor. At this point B cells leave the bone marrow into the periphery where they become naïve B cells, expressing both IgM and IgD. Exposure of the naïve B cell to antigen results in activation and rounds of somatic hypermutation and isotype switching resulting in differentiation to an antibody secreting plasma cell or resting memory B cell.

1.11 Apoptosis and its role in B cell development

As in other cell backgrounds, cell death in B cells can be divided into the disordered cell death or necrosis resulting from acute injury and programmed cell death (autophagy and apoptosis). During autophagy, cell components are degraded by the lysosomal machinery, whereas apoptosis results from irreversible damage caused to cell components through activation of cysteine-aspartic acid proteases (caspases). As shown in Figure 1.9, apoptosis can be identified by a series of distinct morphological changes (Hacker, 2000). During the early stages of apoptosis, cells shrink due to cleavage of actins and lamins and their chromatin condenses. Genomic DNA is then cleaved, organelles condense and blebbing occurs from the cell membrane. Finally the cell disintegrates to form apoptotic bodies; importantly these retain intact cell membranes preventing release of potentially damaging cell contents.

Apoptosis is especially important in B cells for the generation of the B cell repertoire (section 1.10). During B cell development, pre-B cells which fail to produce a functional pre-B cell receptor, immature B cells which react to bone marrow antigens and activated B cells with reduced antigen affinity or crippling mutations acquired during SHM, all die by apoptosis. During B cell development, stimuli which lead to downstream activation of caspases include: inadequate signalling through BCR engagement, insufficient T cell support, inactivation of BCR expression causing the loss of BCR-mediated pro-survival signals and activation of DNA damage checkpoints through accumulation of excessive mutations. However, a large variety of other apoptotic stimuli have also been identified in B cell and non-B cell systems. The vast majority of apoptotic signals lead to activation of caspases through two major pathways; the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway.

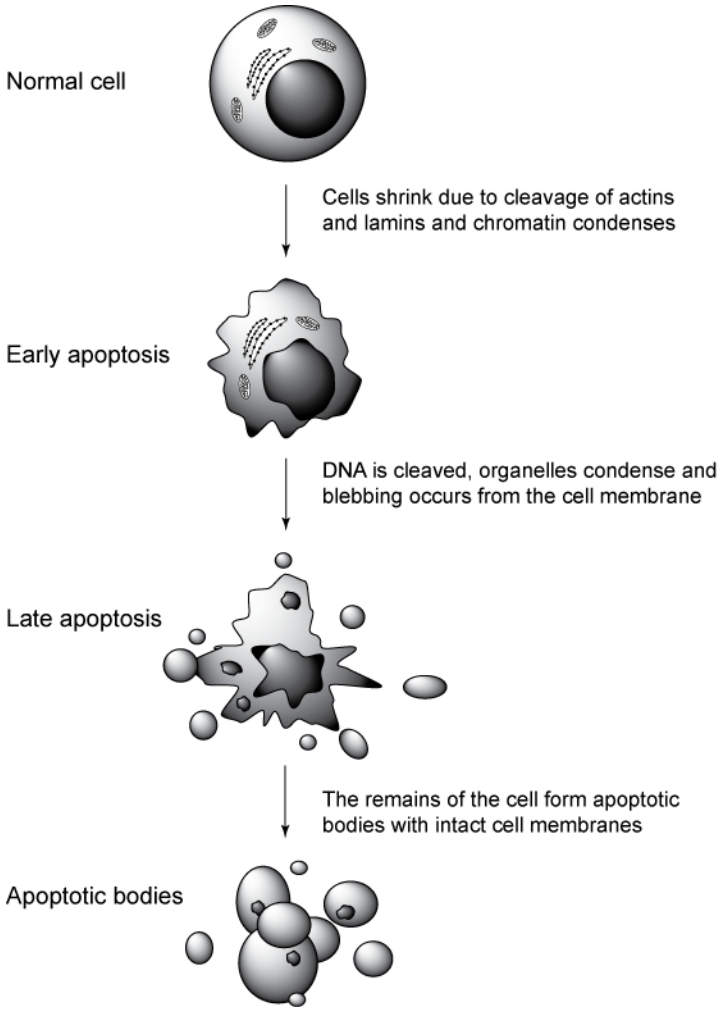


Figure 1.9. This Figure is adapted from Hacker, 2000 and shows the morphological changes associated with apoptosis.

1.11.1 The extrinsic apoptosis pathway

The extrinsic apoptosis pathway is activated by binding of members of the tumour necrosis family (TNF) to their specific tumour necrosis family receptors (TNFR) on the cell surface membrane. Six of these death receptors have currently been identified: TNFR-1 (CD120a), CD95 (APO-1/Fas), DR3 (APO-3, LARD, TRAMP, and WSL1), TRAIL-R1 (APO-2 and DR4), TRAIL-R2 (DR5, KILLER, and TRICK2), and DR6 (Lavrik et al., 2005). Although they have a diverse range of structures, all contain an intracellular death domain (DD) which is essential for transduction of the apoptotic signal. Binding of a death receptor to its appropriate ligand or cross-linking with agonistic antibodies causes the formation of a multimolecular complex of proteins called the death-induced signalling complex (DISC).

Formation of the CD95 DISC is the best characterised of the TNFR activation pathways. As shown in Figure 1.10, binding of CD95 ligand causes trimerisation of the receptor and binding of the DD to the TNFRSF1A-associated death domain protein (TRADD), the receptor interacting protein (RIP) and the serine-phosphorylated adapter Fas-associated death domain protein (FADD). The death effector domain (DED) of FADD in turn binds pro-caspase 8, which is cleaved in the DISC, releasing the activated caspase 8 into the cytoplasm, where it activates the executioner caspases 3 and 7, which are responsible for the late apoptosis markers such as DNA fragmentation and membrane changes. The other death receptors also lead to activation of the executioner caspases through activation of caspases 8 and/or 10 in a similar manner. The exception is TNFα binding to TNFR-1 which has both a pro-apoptotic effect through the recruitment of complex II proteins, TRADD, RIP, FADD and pro-caspase 8 and an anti-apoptotic effect through recruitment of complex I, which contains only TRADD and RIP and activates I-κB kinase (IKK). Activated IKK phosphorylates IκB which releases the anti-apoptotic NFκB protein, which exerts a wide range of cellular effects, including inhibition of caspase 3.

The extrinsic apoptosis pathway is highly important in the clonal selection of B cells. In murine B cells, CD95 is essential for the clearance of self reactive immature B cells from the bone marrow by CD4+T cells (Rathmell et al., 1995). In addition, GC B cells also express high levels of CD95 on their cell surface and, unless stimulated by CD40, die by apoptosis induced by the interaction of FasL with CD95 (Takahashi et al., 2001) and are engulfed by nearby macrophages (Allen et al., 2007; Segundo et al., 1999). Although the extrinsic pathway is clearly key to B cell apoptosis, it also appears that the intrinsic apoptosis pathway plays an important role as clonal deletion of B cells can be delayed by expression of the anti-apoptotic B-cell CLL/lymphoma 2 (Bcl-2) protein (Hartley et al., 1993) and the rapid B cell apoptosis which follows cross-linking of the BCR of mature B cell lines does not require either Fas or FADD proteins (Yoshida et al., 2000).

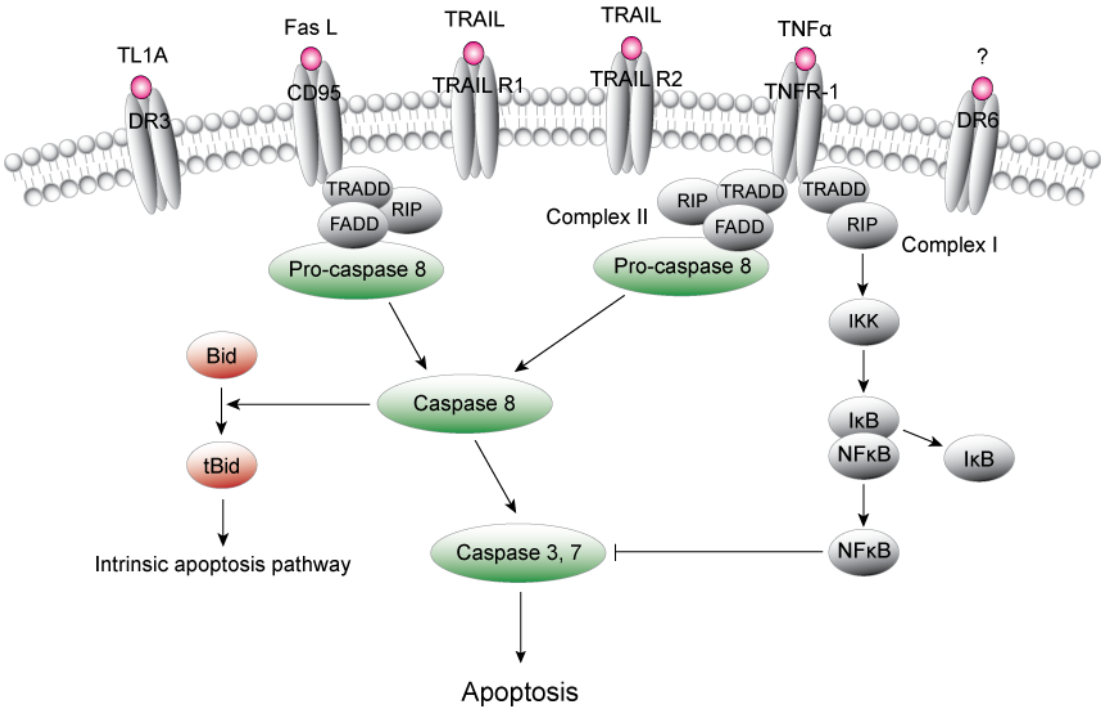


Figure 1.10. This Figure is adapted from Lavrik et al., 2005 and shows a schematic representation of the extrinsic apoptosis pathway. For simplicity, only signalling through death receptors CD95 and TNFR-1 is shown; however signalling through the remaining receptors occurs in a similar manner. Binding of a death receptor to its appropriate ligand results in assembly of the death-induced signalling complex (DISC), which in the case of CD95 contains TNFRSF1A-associated via death domain protein (TRADD), the receptor interacting protein (RIP) and the serine-phosphorylated adapter Fas-associated death domain protein (FADD) and procaspase 8. Inactive procaspase 8 is cleaved within the DISC to the active form and released into the cytoplasm where it activates the executioner caspases 3 and 7, which results in destruction of the cell. Additionally, Caspase 8 also cleaves inactive Bid to active tBid, which activates the intrinsic apoptosis pathway. Binding of TNFα to TNFR-1 either results in the formation TNFR-1 complex II which promotes apoptosis in the same manner as CD95 or results in the formation of TNFR-1 complex I which contains only TRADD and RIP and provides anti-apoptotic signals through activation of NFκB.

1.11.2 The intrinsic apoptosis pathway

A schematic representation of the intrinsic apoptosis pathway is shown in Figure 1.11. The exact mechanisms have not been clarified to the same extent as those involved in the extrinsic pathway, but it is well established that the mitochondrion plays a pivotal role as a sensor to a wide variety of death stimuli (Ow et al., 2008). In response to upstream apoptotic signals, factors such as cytochrome c and Smac/Diablo are released from the mitochondria; cytochrome c combines directly with Apaf-1 and caspase 9 to form the apoptosome, whereas Smac/Diablo acts through inhibition of the inhibitor of apoptosis proteins (IAPs). Formation of the apoptosome leads to activation of caspase 3, which executes programmed cell death.

Key to the regulation of mitochondrial induced apoptosis is the Bcl-2 family member proteins, which possess both pro and anti-apoptotic members and are subdivided into 3 groups by the presence of 4 Bcl-2 homology domains (BH1-4). The anti-apoptotic Bcl-2 family members including Bcl-2, Bcl-XL and Mcl-1 display sequence conservation of all 4 BH regions, whereas pro-apoptotic Bcl-2 proteins are further subdivided into multidomain members (Bax and Bak), which possess homology in BH1-3, and the BH3 only proteins (including Bim, Bad, Bik, Bid, Puma, Hrk and Noxa) (Kim et al., 2006). In viable cells the multidomain pro-apoptotic proteins exist as monomers in the cytosol (Bax) or mitochondria (Bak). Upon death stimulation both Bax and Bak insert directly into the mitochondrial outer membrane (MOM) and through homo-dimerisation facilitate the release of proteins (including cytochrome c and Smac/Diablo) from the intermembrane space (IMS) via a mitochondria apoptosis induced channel (MAC).

Two models currently exist to explain how the activity of the multidomain Bcl-2 family member proteins is controlled. In the direct model the anti-apoptotic proteins (Bcl-2, Bcl-XL and Mcl-1) function by blocking the action of the multidomain pro-apoptotic proteins. Bcl-2 and Bcl-XL have been shown to be capable of heterodimerising with Bak and Bax and preventing their homodimerisation and activation (Antonsson et al., 2001; Sattler et al., 1997). In this model BH-3 only proteins function to induce apoptosis by relieving the activation block imposed on Bax and Bak activity by Bcl-2, Bcl-XL and Mcl-1.

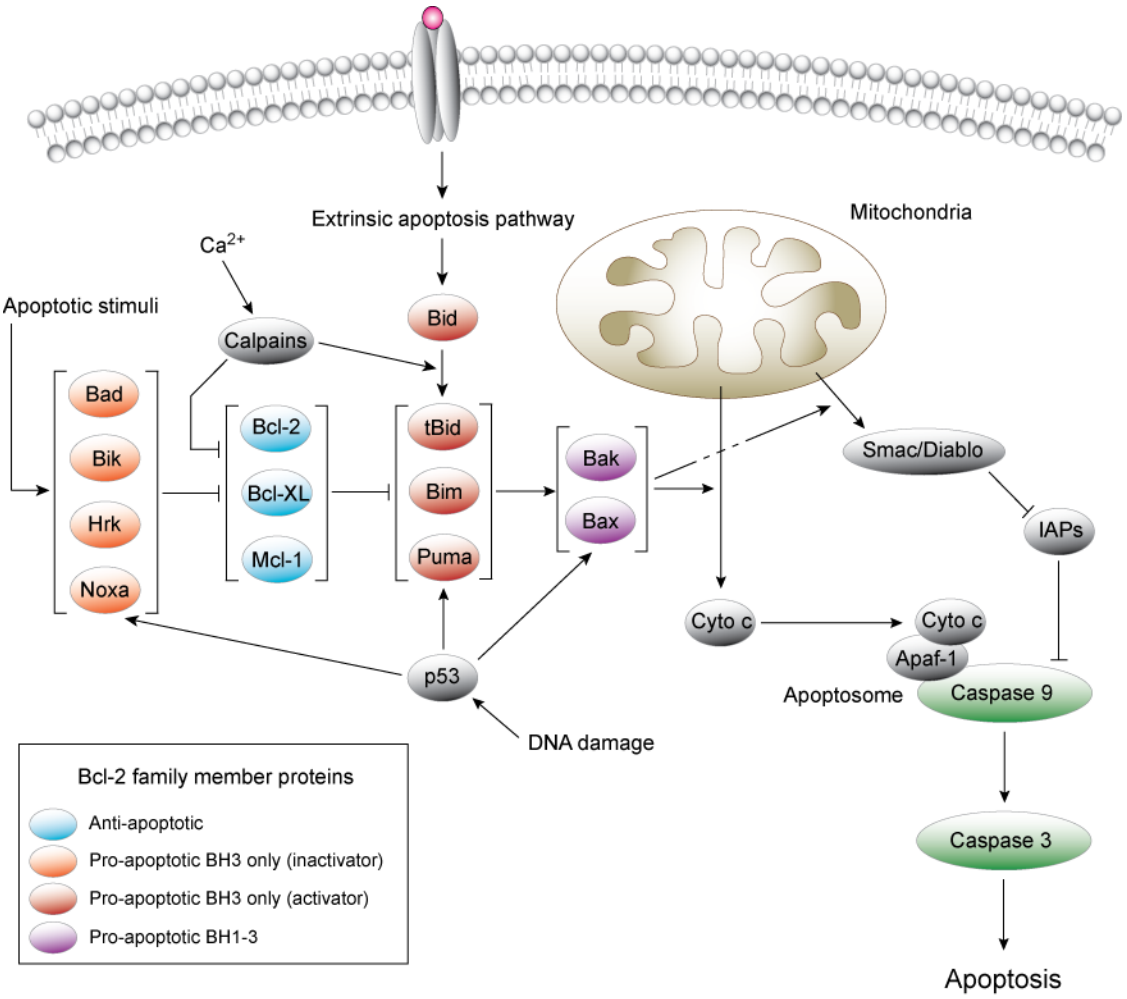


Figure 1.11. This figure is adapted from Kim et al., 2006 and shows a schematic representation of the intrinsic apoptosis pathway. This diagram depicts activation of the pro-apoptotic BH1-3 proteins through the hierarchical model, where inactivator BH3 only Bcl-2 family member proteins inactivate the anti-apoptotic Bcl-2 proteins. This in turn relieves the block on the activator BH3 only Bcl-2 proteins which activate Bak and Bax. Bak and Bax insert into the mitochondrial outer membrane (MOM) and promote the release of smac/diablo and cytochrome c (Cyto c). Cytochrome c then binds Apaf-1 and caspase 9 to form the apoptosome, which then activates caspase 3, while smac/diablo inhibits the anti-apoptotic, inhibitor of apoptosis proteins (IAPs).

However there is increasing evidence for direct activation of Bax and Bak by the BH-3 only proteins tBid, Bim and Puma (Wei et al., 2000; Kuwana et al., 2005). Thus a second hierarchical model for multidomain pro-apoptotic protein activation has been proposed (Kim et al., 2006), where the activity of Bax and Bak is kept in check by cellular factors such as the outer mitochondrial membrane channel VDAC2 (Cheng et al., 2003). It is proposed that Bax and Bak are activated by interaction with "activator BH-3 only proteins" tBid, Bim and Puma. The role of Bcl-2, Bcl-XL and Mcl-1 would be to block these activator BH-3 only proteins, while the activity of the anti-apoptotic Bcl-2 proteins would be in turn modulated by the "inactivator BH3 only proteins". This hierarchical model is presented in Figure 1.11.

A major activator of the intrinsic apoptosis pathway is the tumour suppressor gene (TSG), p53, which detects DNA damage and initiates cell cycle arrest and/or apoptosis. Release of p53 from MDM2 during DNA damage causes p53-mediated transcriptional up-regulation of Bax (Letai et al., 2002), Noxa (Oda et al., 2000a) and Puma (Nakano and Vousden, 2001) and translocation and insertion of Bax into the MOM (Chipuk et al., 2004). Intracellular calcium (Ca^{2+}) is also believed to play a role in activation of the intrinsic apoptosis pathway. Levels of Ca^{2+} exceeding the natural buffering capacity of the cytosol are a well known trigger of apoptosis (Berridge et al., 1998; McConkey and Orrenius, 1997; Orrenius et al., 2003). The exact mechanism of Ca^{2+} induced cell death has yet to be fully elucidated, but treatment of cells with the calcium ionophore, ionomycin, has been shown to activate the calpains, a group of Ca^{2+}-dependent cysteine proteases which are capable of cleaving Bcl-2 family member proteins Bcl-2, Bcl-XL and Bid. Cleavage inactivates the anti-apoptotic Bcl-2 and Bcl-XL proteins and activates the pro-apoptotic Bid protein leading to release of cytochrome c from the mitochondria and activation of caspase 9 (Gil-Parrado et al., 2002).

Finally, there is also a degree of cross talk between the extrinsic and intrinsic apoptosis pathways through the BH3 only protein, Bid. Activation of CD95 by Fas ligand causes proteolytic cleavage of cytosolic Bid to form truncated tBid which is translocated into the mitochondria and interacts with Bak. Interaction of tBid with Bak induces oligomerisation of Bak monomers into a multimeric pore capable of cytochrome c efflux from the mitochondria (Wei et al., 2000).

1.12 Primary EBV infection of B cells *in vivo* and viral persistence

Primary infection by EBV normally occurs through transmission of infectious virus via the salivary route. EBV then establishes a lifelong infection within the memory B cell compartment of the infected individual. Primary infection usually takes place asymptomatically in early childhood (Henle and Henle, 1970); however if infection is delayed until adolescence it may cause development of the self-limiting lymphoproliferative disease, infectious mononucleosis (IM) (Henle et al., 1968). Because infection is normally asymptomatic, much of the evidence for primary infection by EBV comes from IM patients. The primary site of EBV infection was initially believed to be oropharyngeal epithelial cells as virus was detected in epithelial cells from throat washings of IM patients by in situ hybridisation (Gerber et al., 1972; Sixbey et al., 1984; Yao et al., 1985). However, subsequent examination of frozen IM tonsil sections failed to find any EBV-infected epithelial cells (Niedobitek et al., 1997a). Instead they found that EBV was located within the B cells located close to the epithelial crypts (Anagnostopoulos et al., 1995; Karajannis et al., 1997; Tao et al., 1995). It is now believed that viral replication within infiltrating B cells in the oropharynx is essential for primary infection because B-cell–deficient, X-linked agammaglobulinemia (XLA) patients show no evidence of even transient EBV infection in the throat (Faulkner et al., 1999). Although infection of primary epithelial cells *in vitro* using cell free virus is typically very inefficient compared to infection of B cells (Imai et al., 1998), it has recently been discovered that epithelial cells can be readily infected by coculture with B cells with surface bound EBV particles (Pegtel et al., 2004; Shannon-Lowe et al., 2006). This discovery indicates that cell-free virus transmitted through the saliva may first infect B cells, which may then lead to highly efficient transfer infection of epithelial cells. Lytic replication of EBV within epithelial cells could then provide a significant amplification in infectious viral particles available for infection of tonsillar B cells and may also increase viral tropism for B cells, as virus produced in epithelial cells has an increased affinity for the infection of B cells (Borza and Hutt-Fletcher, 2002).

Once B cells within the oropharynx are infected with EBV, they are driven into proliferation by expression of the EBV latent antigens, increasing the pool of infected B cells (Hochberg et al., 2004; Kurth et al., 2003; Niedobitek et al., 1997a). Most cells express the full range of EBV latent antigens including EBNA2 and LMP1; however 2 smaller subsets of infected cells have been identified, one

which expresses only EBNA2 in the absence of LMP1 and a second group which displays an HL-like morphology and expresses LMP1 in the absence of EBNA2 (Kurth et al., 2000). The blood of patients with acute IM also contains large numbers of latently infected B cells which have been reported to express both Latency III associated Cp/Wp initiated transcripts and Latency I associated Qp initiated transcripts, again indicating heterogenic latent antigen expression (Niedobitek et al., 1997a; Tierney et al., 1994). Interestingly, these infected cells are found only in the IgD-negative, CD27-positive memory subset and not the IgD-positive, CD27-negative naïve subset (Hochberg et al., 2004), a trend which was initially identified in the blood of healthy carriers (Babcock et al., 1998).

The reason for this compartmentalisation of infected cells is not known and is the subject of much debate. In one scenario, it has been suggested that during EBV colonization, infection of naïve B cells mimics the process of antigen driven differentiation and drives that cell's progeny to acquire memory status (Souza et al., 2005; Thorley-Lawson, 2001; Thorley-Lawson and Gross, 2004). In mouse and in *in vitro* models, expression of the EBV latent proteins LMP1 and LMP2A appears to be potentially capable of providing the necessary signals to allow an infected B cell to enter a follicle and initiate SHM and class switching, even in the absence of antigen (Casola et al., 2004; He et al., 2003; Souza et al., 2007). An alternative model proposes that the presence of EBV in memory cells is GC-independent and occurs through direct preferential infection of the memory B cell compartment by EBV. It has been reported that the EBV receptor CD21 is expressed at much higher levels on the surface of memory than naïve B cells (Feldhahn et al., 2002); however this remains controversial. Nevertheless, direct infection could explain the apparent lack of infected GC B cells in IM tonsil sections (Anagnostopoulos et al., 1995; Niedobitek et al., 1997a; Weiss and Movahed, 1989). In addition, the persistence of EBV in X-linked lymphoproliferative (XLP) patients (which lack functional GCs) indicates that EBV can colonise a host without to need to transit a GC compartment (Chaganti et al., 2008).

Whichever route EBV utilises to populate the memory B cell compartment, the virus replication and virus driven proliferation and expansion of the B cell pool elicits a strong T cell response. Large numbers of EBV-specific CD8+ CTLs are produced to bring the infection under control, leading to hyperactivation of the immune response and the symptoms of IM (Callan et al., 1998; Sheldon et al., 1973). EBV infections are eventually brought under control and, as IM patients convalesce, virus

replication in the oropharynx decreases and the number of infected peripheral B cells and CTLs falls (Hadinoto et al., 2008). In spite of the strong CTL response, EBV is not completely cleared and after IM EBV remains detectable at low levels both as infectious virus in throat washings and as latent infection in the B cell pool (Miller et al., 1973; Nilsson et al., 1971). Although EBV can frequently be continuously detected at both these sites, full body irradiation during preparation for allogeneic bone marrow transplantation, which completely ablates the B cell compartment but leaves the mucosal epithelium relatively intact, was shown to eradicate EBV from leukemia patents (Gratama et al., 1988). This indicates that the B cell compartment provides the site of persistence while the oropharynx is the site of EBV replication.

To persist within the memory B cell compartment despite the strong CTL surveillance, EBV is forced to adopt a highly restricted form of infection, termed Latency 0 (Babcock et al., 1999). By a mechanism which is not fully understood, EBV down-regulates expression of all latent antigens leaving only the EBERs and BARTs, although low level EBNA1 and LMP2A transcripts have also been detected in some healthy carriers (Chen et al., 1999a; Tierney et al., 1994). As members of the memory pool, EBV-infected cells appear to remain sensitive to physiologic signals and contact with antigen, which promotes differentiation of infected B cells into plasma cells, has been found to activate viral replication (Crawford and Ando, 1986). These lytically active plasma cells tend to localize near mucosal surfaces, such as the oropharynx (Anagnostopoulos et al., 1995), but only a minority complete the EBV lytic cycle, again indicating a possible epithelial role in lytic viral replication (Laichalk and Thorley-Lawson, 2005). Additionally, this occasional lytic replication and the subsequent infection of new B cells serves to replenish the reservoir of infected cells and maintain EBV as a lifelong infection (Babcock and Thorley-Lawson, 2000).

1.13 EBV associated B cell malignancies

In spite of the fact that in healthy carriers EBV infection is limited to memory B cells, EBV has been associated with tumours from a wide range of different B cell developmental stages. In B cell lymphomas, the presence of Ig hypermutations can be diagnostic of the tumour progenitor cell. BL cells, for example, strongly resemble cells of GC origin; they possess rearranged hypermutated Ig genes, often with indications of continuing Ig gene diversification (Klein et al., 1995; Tamaru et al.,

1995). The Ig genes of the Reed-Sternberg cells of HL tumours are also rearranged and hypermutated, but show no indication of continuing Ig gene diversification indicating a post-GC origin (Kuppers et al., 1994), while PTLDs may arise from a range of B cell developmental stages (Carbone et al., 2001).

EBV is also associated with malignancies from other tissue origins, including NK and T cell lymphomas and epithelial carcinomas (NPC). However, as the focus of this thesis is BL, the following sections will focus on tumours of B cells before describing BL in more detail.

1.13.1 Post-transplant lymphoproliferative disease (PTLD)

There is a long established link between immunosuppressive therapy and lymphoma (Gulley et al., 1993) and EBV is well recognised as the causative agent in a large percentage of these post-transplant lymphoproliferative disease (PTLD) cases (Capello et al., 2005; Taylor et al., 2005). PTLD patients commonly present with fever and lymphadenopathy and, upon diagnosis, multifocal lesions are frequently observed within the lymphoid system, the central nervous system, the liver or the transplanted organ (Nalesnik, 1998). The incidence rates differ from 1-5% in renal and liver transplants to 5-15% for heart-lung and small bowel transplants and disease risk correlates with the levels of immunosuppression (Opelz and Dohler, 2004). A second major risk factor is the patient's EBV status prior to transplant, as one study showed a 20-fold increase in incidence of the disease in EBV-seronegative recipients; these patients frequently seroconvert post transplant and have no existing immunity to target infected cells (Ho et al., 1985). The risk associated with preoperative EBV status also explains the increased incidence of PTLD in young children.

PTLDs are classified by the World Health Organisation (WHO) into three histologic types: (a) diffuse B cell hyperplasia lesions, which are usually polyclonal or oligoclonal in origin, occur without disturbance of the normal lymphoid architecture, arise within the first year after transplant and are consistently EBV-positive; (b) polymorphic lesions which may be oligoclonal or monoclonal in origin and are the most common form of PTLD; they occur amid some tumour necrosis and destruction of underlying lymphoid architecture, can appear either early or late after transplant, and are almost always EBV-positive; (c) monomorphic lesions, which are monoclonal in origin; they are classified as diffuse, large B-cell lymphomas as they typically arise several years after transplant and are largely EBV-positive .

Early onset diffuse B cell hyperplastic and polymorphic lesions resemble EBV-infected IM tonsillar B-cell populations in viral gene expression; cells tend to express the full Latency III growth transforming programme indicating that these lesions arise directly from EBV-transformed B cells that grow out in the absence of host T-cell surveillance (Capello et al., 2003; Timms et al., 2003). Where immunoglobulin variable (Ig_V) gene sequences are analyzed, some clones appear to originate from naïve B cells, while others have hypermutated but stable Ig_V sequences consistent with derivation from memory B cells (Brauninger et al., 2003; Timms et al., 2003).

Although some late-onset PTLDs present with similar clinical features to early onset cases, a number of clinical indicators suggest that other cytogenetic changes may promote the onset of these lesions. There is, for example, a much greater heterogeneity in viral protein expression; some tumours express the full range of latent viral antigens, others express only EBNA1, while some express EBNA1 plus EBNA2 and/or LMP1 (Brauninger et al., 2003; Capello et al., 2003; Timms et al., 2003). Other late onset tumours show signs of on-going intraclonal diversification or display Ig_V sequences with crippling mutations, resulting in an unusual BCR-negative B-cell phenotype (Brauninger et al., 2003; Capello et al., 2003; Timms et al., 2003). Such evidence suggests that some late onset tumours arise directly from GC cells or more commonly from atypical survivors of the GC reaction that have been rescued from apoptosis. In these cases, EBV infection, in addition to other events, is likely to have contributed to this escape from GC apoptosis. Accordingly, many monomorphic tumours show mutations of the SHM machinery (Capello et al., 2005) and also frequently contain mutations of p53, bcl-6 or c-myc along with numerous chromosomal aberrations (Djokic et al., 2006; Knowles et al., 1995; Locker and Nalesnik, 1989; Delecluse et al., 1995).

The optimal treatment of PTLD is still a matter of debate; early polyclonal lesions respond well to the reconstitution of EBV-specific T cell control through reduction of immunosuppression, underlying the likelihood that they arise from opportunistic LCL-like outgrowth (Tsai et al., 2001). Changes in immunosuppression are also frequently supplemented with monoclonal antibody therapies such as rituximab (anti-CD20) (Choquet et al., 2006) and CTL transfer therapy (Bollard et al., 2007).

1.13.2 Hodgkin's Lymphoma

Hodgkin's Lymphoma (HL) is one of the most common lymphomas in the Western world, but is unusual as the malignant mononuclear Hodgkin's and multinuclear Reed-Sternberg (H-RS) cells are outnumbered 100-fold by a reactive non-malignant cell infiltrate. Based on the nature of the infiltrate, HL is divided into the nodular sclerosing (NS), mixed cellularity (MC), lymphocyte-predominant (LP) and the rarer lymphocyte-depleted (LD) subtypes (Harris et al., 1994; Kuppers, 2009). Analysis of biopsies by in situ hybridization, Southern blotting and terminal repeat analysis led to the discovery of monoclonal EBV genomes within the malignant H-RS cells of some HL cases (Weiss et al., 1987; Anagnostopoulos et al., 1989; Weiss et al., 1989). Through subsequent analysis of large numbers of HL samples, the association with EBV was shown to vary between the different subtypes; MC and LD tumours show a viral association of 60-90% whereas NS HL has an association of 20-40%. EBV association also varies with age and geographical location. In the developing world, the average association with EBV is around 60-70%. This takes into account childhood HL, which is almost entirely EBV-positive and the adult disease where just over half of all cases are EBV-positive (Ambinder et al., 1993; Hayashi et al., 1997; Weinreb et al., 1996). By contrast, about 35% of HL tumours in the West are EBV-positive and this can be divided into an incidence of 50% in children and 30% in adults (Glaser et al., 1997). The high frequency of EBV-positive disease in children in developing countries, where primary infection occurs early in life, indicates an increased risk of HL soon after exposure to EBV. This trend is also displayed in the West where delayed infection resulting in IM confers a fourfold risk of subsequently developing the EBV-positive form (Hjalgrim et al., 2003). In EBV-positive HL, all H-RS cells express a Latency II pattern of viral gene expression characterised by Qp-driven EBNA-1, and expression of LMP1, LMP2A, LMP2B, EBER and BARTs from their own promoters (Deacon et al., 1993). The presence of EBNA1, LMP1 and LMP2A protein in H-RS cells has also been confirmed by monoclonal antibody staining (Grasser et al., 1994; Herbst et al., 1991; Niedobitek et al., 1997b).

The origin of the malignant H-RS cells remained enigmatic for decades as neither EBV-positive nor EBV-negative H-RS populations express conventional lymphocyte markers. Microdissection of individual H-RS cells followed by PCR amplification of the Ig gene revealed that nearly all H-RS cells within a single tumour show the same Ig heavy and light chain gene rearrangements, indicating that HL is of monoclonal B-cell origin (Braeuninger et al., 1997; Kanzler et al., 1996; Kuppers et al., 1994;

Muschen et al., 2000). Further analysis of the Ig_V regions revealed the hallmarks of SHM. Thus H-RS cells must have transited through a GC reaction, a process which would normally be dependent on functional BCR expression (Goossens et al., 1998). Interestingly however, most cases of classical HL lack Ig expression and 25% contain crippling mutations resulting in stop codons within the ORF (Kuppers et al., 1994; Nonoyama et al., 1973). The presence of these crippling mutations in the absence of ongoing Ig gene mutation suggests that H-RS cells have been rescued from a fate of apoptosis within the GC (Weiss et al., 1992).

Microarray analysis of both EBV-positive and EBV-negative H-RS cells showed a down-regulation of B cell specific genes (Hartmann et al., 2008; Kuppers et al., 2003; Schwering et al., 2003), likely to be driven by expression of the helix-loop-helix proteins ABF-1 and Id2 (Mathas et al., 2006). H-RS cells also show constitutive activation of the Notch 1, STAT and NFκB signalling pathways and aberrant expression of the inhibitor of apoptosis (IAP) family member protein, XIAP (Brauninger et al., 2006). NFκB activity contributes both to continued cell growth (Bargou et al., 1997) and resistance to extrinsic Fas-mediated apoptosis through binding of NFκB-regulated c-FLIP protein to the CD95 DISC which prevents the binding of procaspase 8 (Dutton et al., 2004; Thomas et al., 2002). Elevated XIAP levels simultaneously block intrinsic apoptosis signals by preventing the formation of the apoptosome (Kashkar et al., 2003).

In EBV-positive HL cases it is highly likely that the EBV antigens LMP1 and LMP2A are responsible for the rescue of GC B cells from apoptosis (Bechtel et al., 2005; Chaganti et al., 2005). As discussed in section 1.8.5, LMP1 mimics many of the signals normally delivered to B cells through ligation of cell surface CD40 (Mosialos et al., 1995) leading to constitutive TRAF signalling and activation of the downstream NFκB, JNK and p38 pathways (Dirmeier et al., 2005; Henderson et al., 1991). Likewise, LMP2A (section 1.8.6) can mimic the effects of antigen binding and deliver BCR-like survival signals to B cells by recruiting the src family tyrosine kinases (Caldwell et al., 1998). Such a scenario is supported by comparison of clonal Ig_V region sequences in HL cases to the EBV status of the tumour, which showed that almost all tumours with fully crippling mutations or deletions were EBV-positive (Brauninger et al., 2006).

1.13.3 Burkitt Lymphoma

Endemic BL (eBL), as first described by Denis Burkitt (Burkitt, 1958), is the most common childhood cancer in equatorial Africa, but has a highly unusual pathogenesis which is linked both to EBV and to the activation of the cellular oncogene, c-myc. In contrast to PTLDs, EBV-positive BL tumours express only the EBNA1 protein along with the non-coding EBERs and BARTs (Rowe et al., 1987) and a collection of EBV encoded miRs (Cai et al., 2006).

More recently two further forms of BL have been discovered, namely sporadic BL and AIDS-associated BL. All three subtypes share a similar histologic appearance, with a malignant population of small round monomorphic B cells, interspersed with macrophages, which give the tumour a characteristic 'starry sky' pattern (Kelly and Rickinson, 2007). All three forms of BL have a cell differentiation phenotype closely resembling that of normal centroblasts, the cells populating the proliferative compartment of GCs. BL tumour cells are bcl-6 positive and express centroblastic cell surface markers, CD10 and CD77; this phenotypic similarity between BL and GC cells has subsequently been confirmed by microarray analysis of cellular gene transcription (Kuppers et al., 2003). Individual tumours are monoclonal in terms of Ig gene rearrangement and isotype expression; most are surface IgM-positive, usually without IgD; however some tumours express IgG or IgA. Sequencing of the productively rearranged heavy and light chain genes of BL cells confirmed that they are hypermutated, strengthening the likelihood that cells have transited a GC reaction and a small number of tumours also showed some ongoing Ig diversification during serial passage *in vitro* (Chapman et al., 1995; Klein et al., 1995; Tamaru et al., 1995). The epidemiological features of the three different BL subtypes are shown in Table 1.

Form of BL	Geographic location	Incidence per 100,000/y	Cofactors	% EBV association
Endemic (eBL)	Equatorial Africa and Papua New Guinea	5-10, for age 3-12 y	Holoendemic malaria	100
Sporadic	Worldwide	10 -100-fold less than e-BL	Currently unknown	15-85 (varies with location)
AIDS-associated	Worldwide	10-100-fold more than e-BL	HIV	30-40

Table 1. This table is adapted from Kelly and Rickinson, 2007 and shows the epidemiological features of the three different subtypes of BL. Data for the incidence rates comes from Africa for eBL, worldwide for sporadic BL and from untreated AIDS cohorts in the early years of the AIDS epidemic in the U.S. and Europe for AIDS-associated BL.

Endemic BL (eBL) is the most common of the BL subtypes and is found exclusively in areas of equatorial Africa and Papua New Guinea which are holoendemic for Plasmodium falciparum malaria (Burkitt, 1962b; Burkitt, 1962a; Rainey et al., 2007a; Rainey et al., 2007b). Within these areas there is an incidence rate within 3-12 year olds of 5-10 cases per 100,000 per year with a male to female ratio of 3:1. eBL tumours commonly arise at unusual sites including the jaw, the orbit of the eye or the ovaries. Virtually all eBLs are EBV-positive and almost all tumours carry monoclonal EBV episomes within every cell (Epstein et al., 1964b; Magrath, 1990; Raab-Traub and Flynn, 1986).

When compared with the endemic form, the incidence rate for sporadic BL is roughly tenfold lower in North Africa and South America and 50-100-fold lower in Europe and the United States. In addition, sporadic BL usually presents as an abdominal mass and the median age of diagnosis is slightly older (Magrath, 1990). The association of sporadic BL with EBV varies with geographical location. In Western countries only 15-20% of tumours are EBV positive; however in other locations, such as equatorial areas of Brazil, the association with EBV is up to 85%. The incidence of sporadic BL correlates broadly with the association with EBV; thus it appears there is a low base-line incidence of EBV-negative BL in children worldwide, which in some areas is complemented by a variable number of EBV-positive cases (Magrath, 1990). It is therefore possible that in sporadic BL other as yet unidentified parasitic infections may increase tumour incidence and that this preferentially involves the EBV-associated disease form.

A third, AIDS-associated adult form of BL was discovered in the 1980s with identical histology and similar sites of presentation to the sporadic form. AIDS-associated BL is unusually common in those infected with human immunodeficiency virus (HIV) and accounts for 30-40% of all AIDS lymphoma cases. In contrast to the PTLD-like lymphomas that arise in late stage HIV infection, AIDS-associated BL tends to appear early in disease progression when patients are still relatively immunocompetent (Carbone, 2003).

A feature common to all subtypes of BL is high and deregulated c-Myc expression, which results from a reciprocal translocation that places the c-myc gene under the control of the Ig loci. The translocations involve chromosome 8, at or near the site of the c-myc locus at 8q24 and either the Ig_H chain locus (termed the 'common' translocation because it is present in more than 80% of tumours) or the Ig_L κ or λ chain loci (termed 'variant' translocations). Most work has focused on the common (8:14) translocation, where the position of the chromosomal breakage both within c-myc and within the Ig_H loci show interesting differences between the BL subtypes. In general, breakpoints in eBLs occur more than 100kb upstream of the first exon of the c-myc gene and in the VDJ region of the Ig_H locus. In contrast, sporadic and AIDS-associated BL breakpoints commonly occur within the first exon or intron of the c-myc gene and within class switch regions of the Ig_H locus, possibly indicating that the translocation occurred at a different stage of B cell differentiation in these tumours (Joos et al., 1992; Magrath, 1990; Pelicci et al., 1986; Shiramizu et al., 1991). The mechanisms behind c-myc translocations are not fully understood, but it is postulated that they may arise due to mistakes during the maturation of antibody responses in GCs (Kuppers, 2003; Kuppers, 2005). This hypothesis could explain the role of holoendemic malaria and infection with HIV as risk factors for BL development. Holoendemic malaria is known to act as a chronic stimulus to the B-cell system and, by analogy to other parasitic infections (Araujo et al., 1999), it is likely to increase GC activity. In addition, malaria may also contribute to the development of eBL tumours through suppression of EBV-specific T cell responses (Geser et al., 1989; Gunapala et al., 1990; Moormann et al., 2007). This T cell suppression may be responsible for the very high EBV loads observed in the blood of children living in holoendemic malarial areas (Moormann et al., 2005; Rasti et al., 2005) and the even higher level of EBV DNA seen during acute malarial infection (Njie et al., 2009; Yone et al., 2006).

In a similar manner, infection with HIV also acts as a chronic stimulus to the B-cell system. The EBV load within the latently infected B cells of HIV patients increases long before any obvious T-cell impairment, (Piriou et al., 2004) and during the same period of infection patients often develop persistent generalised lymphadenopathy. Histologic analysis of such lesions reveals lymphoid hyperplasia with greatly expanded GC regions which will clearly increase the number of cells at risk of c-myc translocation (Ioachim et al., 1990).

1.13.3.1 Cellular gene expression in BL tumours

Gene expression profiling provides an opportunity to measure the expression of several thousand genes in multiple cell backgrounds in order to determine global patterns of gene expression. This technique has previously been used to demonstrate that BL cells (regardless of EBV status) display a GC-like pattern of gene expression (Kuppers et al., 2003). More recently, gene expression profiling has been used in 2 studies to compare the cellular gene expression of BL with diffuse large B cell lymphoma (DLBCL) (Dave et al., 2006; Hummel et al., 2006), two tumours which in some cases have a significant degree of overlap in their histological presentation. The distinction between these pathogenetically distinct entities is clinically important, as the low dose cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) chemotherapeutic regimens used for DLBCL are ineffective at treating BL (Bishop et al., 2000; Butler and Hainsworth, 1993) for which intensive chemotherapy is required (Magrath et al., 1996; Pees et al., 1992; Divine et al., 2005).

Hummel et al., (2006) analysed the gene expression of 220 mature aggressive B-cell lymphomas which had previously been examined by an expert panel of pathologists. Included among these samples was a core group of 8 BL samples which met all of the WHO histological criteria for BL, which they used to develop a molecular signature for BL. The expression profiles of 105 non-core group samples were sequentially compared to the core group using a method termed “core group extension”. This method assigned each new sample an index of “Burkitt-Likeness” ranging from 0-1, indicating a probability that the sample belonged to the core group. Samples with a score greater than 0.95 were determined to be molecular BLs (mBL), samples with a score less than 0.05 were designated as non-mBL and the remaining cases were considered to be intermediate. The genes which make up the mBL signature were selected based on their contribution to the classification of

samples as mBL or non-mBL. A large number of models were investigated using various statistical permutations and a final model was selected based on its ability to successfully assign samples to the mBL or non-mBL groups and to correctly identify the core group as mBL samples. When used on the remaining 107 samples, this model accurately identified conventional BL samples and also identified the mBL signature in several samples initially identified by conventional histology as unclassifiable mature aggressive B-cell non-Hodgkin's Lymphoma, atypical BL or DLBCL. The parallel study by Dave et al., 2006 used 25 histologically verified cases of BL from their 303 sample archive to develop a similar statistical algorithm also capable of differentiating BL from DLBCL and which also identified the overlap between BL cells and GC B cells. As before, they found the mBL signature in several unidentified high-grade lymphomas, atypical BLs and DLBCLs.

Both studies also investigated chromosomal abnormalities by fluorescence in situ hybridization (FISH) and array based comparative genome hybridization. They found that most cases of molecularly defined BL carried the characteristic c-myc/Ig gene translocation, but relatively few, if any, additional genetic changes. The combination of gene expression and genetic analysis also revealed that, although high c-Myc expression is an integral part of the mBL signature, a small number of tumours molecularly defined as BLs lacked such a translocation. In these rare cases the tumour may arise as a result of c-Myc deregulation by another mechanism such as loss of miRs which ordinarily target and suppress c-myc expression (Leucci et al., 2008).

Full gene expression profiling is currently not a cost-efficient diagnostic option; however this work identified mBL signature genes which can be assayed by immunohistochemistry such as Tcl1 and Bcl6 (both highly expressed in mBLs) and CD44 and MUM1 (both absent from mBLs) which could augment markers currently in use. Currently the mBL signature has only been investigated in sporadic EBV-negative BL samples, but it would certainly be informative to investigate if the expression of the mBL signature genes is affected by BL subtype or EBV status.

1.13.4 Role of c-Myc in the pathogenesis of Burkitt Lymphoma

The c-myc proto-oncogene is a transcriptional regulator which controls numerous cellular processes including proliferation, differentiation and apoptosis (Dang, 1999). *In vitro*, c-Myc is virtually undetectable in quiescent cells, but following mitogenic or serum stimulation c-Myc mRNA and protein

are rapidly induced and remain at a constant level during the cell cycle (Amati et al., 1998; Eilers, 1999); (Amati, 2001). Once expressed the mature protein has a half life of only 30 minutes (Hann and Eisenman, 1984; Spencer and Groudine, 1991), thus protein levels can be tightly regulated by external stimuli.

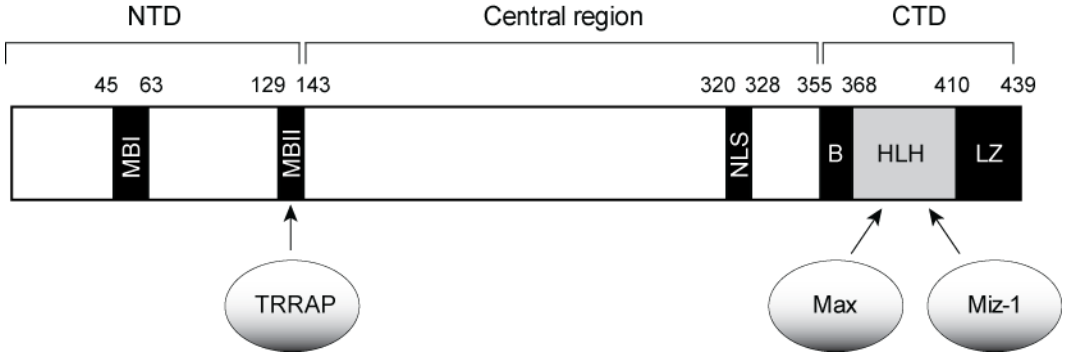


Figure 1.12. Schematic representation of the major domains of the c-myc protein. Contained within the amino-terminal domain (NTD) are the Myc Box (MB) I and II; the central region contains a nuclear localisation sequence (NLS) and the carboxyl -terminal domain (CTD) contains the basic helix loop helix zipper (bHLHZ) domain which is comprised of a basic (B) sequence, a helix loop helix (HLH) and a leucine zipper (LZ).

The 439 aa c-Myc protein (Figure 1.12) contains 2 domains essential for its many functions. The amino-terminal domain (NTD) of c-MYC is responsible for the transactivation of target genes and contains two highly conserved Myc Box (MB) elements (MBI and MBII), while the carboxyl-terminal domain (CTD) contains a basic helix loop helix zipper (bHLHZ) domain which is responsible for binding of c-Myc to its partner bHLHZ protein, Max. Myc-Max heterodimers bind the E-box sequence CACGTG (Amati et al., 1992; Amati et al., 1993a; Amati et al., 1993b) and stimulate the expression of large numbers of genes including the translation initiation factors eIF-4E and eIF-2α, which are important in cell growth (Rosenwald et al., 1993; Jones et al., 1996) and cyclin D2 and CDK2, which are essential for cell-cycle progression (Steiner et al., 1995; Berns et al., 1997). c-Myc is also capable of gene activation through chromatin remodelling via the association of MBII with transformation/transcription domain-associated protein (TRRAP). This co-activator is a component of a large complex that contains histone acetyltransferase (HAT) activity (McMahon et al., 1998; McMahon et al., 2000)). Although c-Myc predominantly functions through transcriptional activation, it has also been shown to act as a transcriptional repressor. While there are likely to be several means of

repression by c-Myc (Kleine-Kohlbrecher et al., 2006), at least one mechanism involves specific binding of the transcriptional activator Miz-1 which also binds the bHLHZ domain of c-Myc. Miz-1 binding has been implicated in the repression of the cyclin-dependent kinase inhibitor (CKI), p21, which again contributes to cellular proliferation (Wu et al., 2003).

c-Myc's pro-proliferative effects are counterbalanced by Mad protein, which also forms heterodimers with Max and down-regulates Myc-induced gene transactivation (Ayer et al., 1995; Grandori et al., 2000; Schreiber-Agus et al., 1995). Overexpression of c-Myc also activates apoptotic checkpoints, including the p53 programme which is activated through the nucleolar tumour suppressor, ARF (Eischen et al., 1999). In lymphoid cells, c-Myc has also been shown to down-regulate expression of the anti-apoptotic Bcl-2 family member proteins, Bcl-2 and Mcl-1 (Eischen et al., 2001; Maclean et al., 2003) and up-regulate expression of the pro-apoptotic Bim and Bax proteins (Juin et al., 2002; Mitchell et al., 2000).

Deregulation of c-Myc has been implicated in a range of human cancers including BL where a reciprocal c-myc translocation places the c-myc gene under the control of the Ig loci. Investigation of genomic c-myc binding sites in BL cells using microarray technology found c-myc bound to nearly 15% of gene loci (Li et al., 2003). These global c-Myc induced changes in gene expression are responsible for the high rate of proliferation observed in BL cells but are also reflected in a high rate of apoptosis.

In an attempt to understand the mechanisms of c-myc driven tumourigenesis in BL cells, an *in vitro* reconstruction system was developed by infecting B cells with a recombinant EBV whose transforming function is oestrogen-dependent. Transfection of the resultant LCLs with a c-myc expression vector, cloned from a BL c-myc/Ig fusion locus, rendered cell growth independent of oestrogen. Furthermore oestrogen-independent cells had down-regulated LCL-associated cell activation and adhesion molecules and up-regulated BL-associated germinal centre markers (Polack et al., 1996; Schuhmacher et al., 1999; Pajic et al., 2001). This work suggests that c-myc overexpression can impose a germinal centre phenotype in B cells despite the fact that normal germinal centre cells lack c-myc expression. This raises the possibility that the GC phenotype of BL cells may be a property of c-myc expression and not a reflection of the true origin of BL cells (Klein et al., 2003).

Several attempts have also been made to recapitulate the malignant BL phenotype in mice using Ig-myc fusion transgenes. Fusion of c-myc to the Ig_H enhancer resulted in tumourigenesis, but lesions did

not resemble classical BL and were found to be of pre-B cell origin (Adams et al., 1985). Subsequent work, using a mutated c-myc translocated to the Ig_L enhancer from a naturally occurring case of BL, produced mouse B-cell tumours with a histological and phenotypic appearance closely resembling BL (Kovalchuk et al., 2000; Zhu et al., 2005). This requirement for mutations within c-myc may reflect a need to impair the apoptotic signal, while retaining proliferative function (Dang et al., 2005; Hemann et al., 2005). Indeed, analysis of c-myc sequences within BL tumours found mutation of the MBI T58 residue in around 20% of BL cases (Adhikary and Eilers, 2005). This mutation is believed to prevent c-myc targeting for proteasomal degradation and inhibit expression of the pro-apoptotic Bim protein (Hemann et al., 2005). BL tumours which have a WT c-myc frequently have mutations of p53 or ARF or overexpress the p53 degradation targeting protein, MDM2 (Eischen et al., 1999; Lindstrom et al., 2001). Thus activation of both Bim and p53 may be required for c-Myc induced apoptosis and therefore disruption of either one of these mechanisms would be sufficient to protect cells from c-Myc induced apoptosis (Dang et al., 2005). It is also possible that in many cases of BL (especially eBL), EBV may be able to offer protection from c-Myc induced apoptosis; however this possibility has yet to be fully examined.

1.13.5 Role of EBV in the pathogenesis of Burkitt Lymphoma

The precise role of EBV in BL remains enigmatic; however the high frequency of EBV association with BL (particularly the endemic form) strongly implies a role for the virus in tumour pathogenesis. Indeed, eBL patients have unusually high antibody titers against the EBV capsid antigen (VCA) and in a large prospective survey in Uganda, children who developed BL had significantly raised anti-VCA months or years before the clinical onset of the tumour (de The et al., 1978; Geser et al., 1982). The oncogenic potential of EBV is also evident in the ease with which it transforms B cells *in vitro* into permanently proliferating LCLs. Interestingly however, BL tumours lack expression of nearly all latent antigens, including the major EBV growth transforming proteins, EBNA2 and LMP1. As described in section 1.7.2, most BL tumours express a highly restricted form of viral gene expression termed Latency I. Cells express only the EBNA1 protein from the Q promoter along with the non coding EBERs, the BARTs and a selection of virally encoded microRNAs. As expression of these transcripts alone appears inadequate for oncogenesis, it has been postulated that BL progenitor cells may initially

express the potent Latency III growth transforming programme observed in LCLs. This could initiate pathogenesis by expanding the population of cells at risk for translocation or by increasing the survival or proliferation of a translocation-positive but still not fully malignant clone. At some stage during the lymphomagenic process, viral transcription would then switch to Latency I as accumulated cellular changes render cell growth independent of viral-transforming proteins. Down-regulation of the growth transforming programme may also result from the incompatibility of deregulated c-Myc expression and Latency III gene expression (Pajic et al., 2001), which may result from the opposing effects on transcription of c-Myc and the EBNA2 protein. In addition, high myc levels may also protect cells from recognition by CD8+ CTL through down-regulation of components of the immunoproteasome (Bayliss and Wolf, 1981) and TAP peptide transporter (Rowe et al., 1995).

A second form of myc compatible viral gene expression has been recently identified in a subset (~15%) of BL tumours (Kelly et al., 2002; Kelly et al., 2006). Termed 'Wp restricted tumours' because of the activation of the Latency III associated Wp promoter and silence of the other viral promoters, these BL cells express EBNA 1, 3A, 3B, 3C, a truncated EBNA-LP, the early lytic cycle gene, BHRF1 and the non-coding EBERs. A key determinant of this alternative latency programme is the presence of an EBNA2 deleted virus genome within the tumour cells. Wp-restricted latency is therefore likely to have arisen from a rare mutational event which provides a selective advantage *in vivo* rather than from a perturbation of normal EBV biology. Accordingly, comparison of Wp-restricted BL cells with conventional Latency I lines has shown that the broadening of EBV gene expression to include the EBNA3s offers the cells significant additional protection from a variety of apoptotic triggers (Kelly et al., 2005). The source of this protection from apoptosis appears to derive from a down-regulation of the pro-apoptotic Bim protein (Leao et al., 2007). Down-regulation of Bim may also negate the need for mutation of the ARF-MDM2-p53 tumour suppressor pathway, disruption of which is frequently observed in mouse B-cell lymphomas with a c-Myc Ig translocation (Herbst et al., 1991; Schmitt et al., 1999) and in most sporadic and eBL tumours (Farrell et al., 1991; Bhatia et al., 1992; Lindstrom et al., 2001).

It would appear from the consistency with which c-myc gene translocation is linked to all forms of BL, from the dominance of c-myc on the global expression in BL cells and from the ability of translocation-based c-myc expression constructs to impose the classic BL-cell phenotype on B cells, that the c-myc

deregulation is the primary factor driving BL pathogenesis. EBV, however, is likely to be an important co-factor in the a etiology of large numbers of BL cases and (as discussed below) is likely to play a continuing role in BL cell survival.

1.14 Loss of EBV from Burkitt Lymphoma cells in culture

There is considerable circumstantial evidence that EBV acts as more than a passenger in proliferating BL tumours and may indeed complement deregulated c-myc expression. Virtually all EBV-positive BL tumours carry monoclonal EBV genomes within every cell (Magrath, 1990; Raab-Traub and Flynn, 1986) and when cultured *in vitro*, most EBV-positive BL cell lines retain EBV and are unable to tolerate loss of the virus (Nasimuzzaman et al., 2005). The observation that rare cells from Akata-BL (an EBV-positive Latency I, sporadic BL) cell line spontaneously lose the virus during late passage culture provided an interesting opportunity to directly observe the effect of EBV in proliferating BL cells (Shimizu et al., 1994). Isogenic EBV-positive and novel 'EBV-loss' clones were generated from Akata-BL by limiting dilution and their comparison revealed that loss of Latency I EBV infection rendered cells unable to grow in low serum concentrations or soft agar and reduced their tumourigenicity in nude mice. The fact that EBV was only lost from BL cells after prolonged periods (around 2 years) in culture suggests that additional cytogenetic changes may have been required to alleviate the absolute requirement for EBV.

Subsequent work using the same system revealed that loss of EBV also caused a modest but significant reduction in resistance to UV irradiation, cycloheximide and glucocorticoid induced apoptosis, while reinfection of EBV-loss clones with a recombinant virus demonstrated that EBV was the causative agent for this phenotype (Komano et al., 1998). Similar observations have since been made using endemic BL lines, which in rare cases yielded EBV-loss clones from cells in late passage (Kitagawa et al., 2000) and more recently from early passage cells (Kelly et al., 2006). The mechanism of apoptosis protection is still very much under debate; an increase in the expression of the anti-apoptotic Bcl-2 and/or a cell cycle-dependent down-regulation of the c-myc protein were suggested as possible mechanisms (Komano et al., 1998; Ruf et al., 1999) and a more recent study implicated the proto-oncogene, Tcl-1 (Kiss et al., 2003). Reduced expression of Tcl-1 was found in both endemic and

sporadic BL cell lines following loss of EBV and expression could be restored by reinfection with recombinant EBV.

1.15 Possible role for Latency I transcripts in EBV-positive phenotype

A number of EBV proteins such as EBNA2, LMP1, BHRF1, BALF1 and vIL-10 have previously been linked with protection against apoptosis. However, as described in section 1.7.2, most EBV-positive BL cells express only the EBNA1 protein, the non coding EBERs, the BARTs (where protein coding capacity has yet to be fully analysed) and a selection of virally encoded microRNAs. Discussed below is the evidence for each of these viral products in the cellular phenotype associated with Latency I infection.

1.15.1 EBNA1

EBNA1 would appear to be a good initial candidate for EBV-mediated protection from apoptosis as it is the only viral protein expressed in EBV-positive Latency I BL cell lines (Rowe et al., 1987). The tumourigenic potential of EBNA1 has been investigated using transgenic mouse models. In an initial study, EBNA1 was expressed in mice under the control of the Ig_H enhancer and the polyoma promoter and backcrossed into the C57BL/6J background where it appeared to promote B cell lymphoma (Wilson and Levine, 1992; Wilson et al., 1996). However a subsequent study, where EBNA1 was expressed under the control of the Ig_H promoter and enhancer in FVB mice, found that EBNA1 was unable to induce tumours and had no effect on mouse mortality, body weight or any lymphocyte subset (Kang et al., 2005). A backcross of these mice into the C57BL/6J background produced similar results (Kang et al., 2008), disputing the initial findings in the C57BL/6J mouse background and indicating that tumourigenesis in the initial study may have been due to the transgene insertion.

It is also possible that EBNA1 could alter cell growth by enhancing expression from EBNA1 cognate sequences in cellular DNA or by interacting with a cellular protein. Expression of a dominant negative EBNA1, which blocks the interaction of WT EBNA1 with cellular DNA, appeared to have no effect on cellular gene expression or LCL cell growth (Kang et al., 2001). However in BL cell lines a dominant negative EBNA1 appeared to reduce viability of EBV-positive BL cells, but not EBV-negative lines (Kennedy et al., 2003). Expression of EBNA1 also appeared able to rescue cells from apoptosis

induced through expression of p53. A possible mechanism for this EBNA1-mediated protection from p53-mediated apoptosis may lie with its ability to bind the p53 regulatory protein USP7 (Saridakis et al., 2005). Interaction of p53 with USP7 deubiquitinates and stabilises the p53 protein, which increases cellular p53 levels and reduces cell viability. EBNA1 binds the p53 binding region of USP7 more extensively than p53, raising the possibility of p53 displacement from USP7 by EBNA1. However, the significance of this finding for the survival of BL tumours remains unclear as the majority of BL cell lines carry aberrations of the p53-MDM2 pathway which would render any modulation of p53 levels immaterial (Farrell et al., 1991; Bhatia et al., 1992; Lindstrom et al., 2001).

Perhaps the most convincing evidence against EBNA1 as a mediator of cell growth and survival comes from the initial investigation of EBV-loss from BL cells, where transfection of EBNA1 into EBV-loss clones was unable to restore the EBV-positive phenotype (Komano et al., 1998; Ruf et al., 1999). This suggests a role for one of the other Latency I-associated gene products.

1.15.2 EBERs

As described in section 1.8.7, the EBERs appear to significantly enhance the B cell transforming activity of EBV *in vitro* (Wu et al., 2007; Yajima et al., 2005). In the Akata-BL system EBERs have been reported to be responsible for the EBV-mediated increase in survival in low serum and soft agar, tumourigenicity in SCID, Bcl-2 up-regulation and protection from apoptosis (Komano et al., 1999). However an independent study found only a partial restoration of the EBV-positive phenotype, possibly related to an inability of EBERs to elicit the down-regulation of c-myc levels observed in EBV-positive cells (Ruf et al., 1999; Ruf et al., 2000).

Figure 1.13 shows the two major mechanisms by which EBERs are reported to enhance BL tumourigenicity and apoptosis resistance. Firstly, EBERs were shown to protect cells from apoptosis induced by IFNα through an EBER-mediated inhibition of the antiviral RNA-dependent protein kinase, PKR (Nanbo et al., 2002; Nanbo et al., 2005). Binding of double-stranded viral RNA ordinarily induces phosphorylation and dimerisation of PKR leading to inhibition of protein synthesis and induction of apoptosis through the translation initiation factor eIF2α (Kitajewski et al., 1986; Dar et al., 2005; Dey et al., 2005). However, despite evidence that EBERs clearly interact with PKR in a cell free system (Sharp et al., 1993; Vuyisich et al., 2002), the role of PKR in EBER-mediated protection from

apoptosis remains controversial. EBERs have yet to be detected in association with PKR in latently infected cells and use of phosphorylation specific antibodies revealed that EBERs were unable to prevent phosphorylation of PKR during IFNα treatment (Ruf et al., 2005). In addition a study using an EBER KO virus also found that the presence of EBERs had no effect on the ability of EBV to transform B lymphocytes in the presence of IFN, the sensitivity of established LCLs to IFNα or the replication of IFN sensitive vesicular stomatitis virus within EBV-transformed lymphocytes (Swaminathan et al., 1992). Further investigation in cell free systems also revealed that binding of EBER1 to the ubiquitous ribosomal protein, L22, attenuates its ability to associate with PKR (Elia et al., 2004).

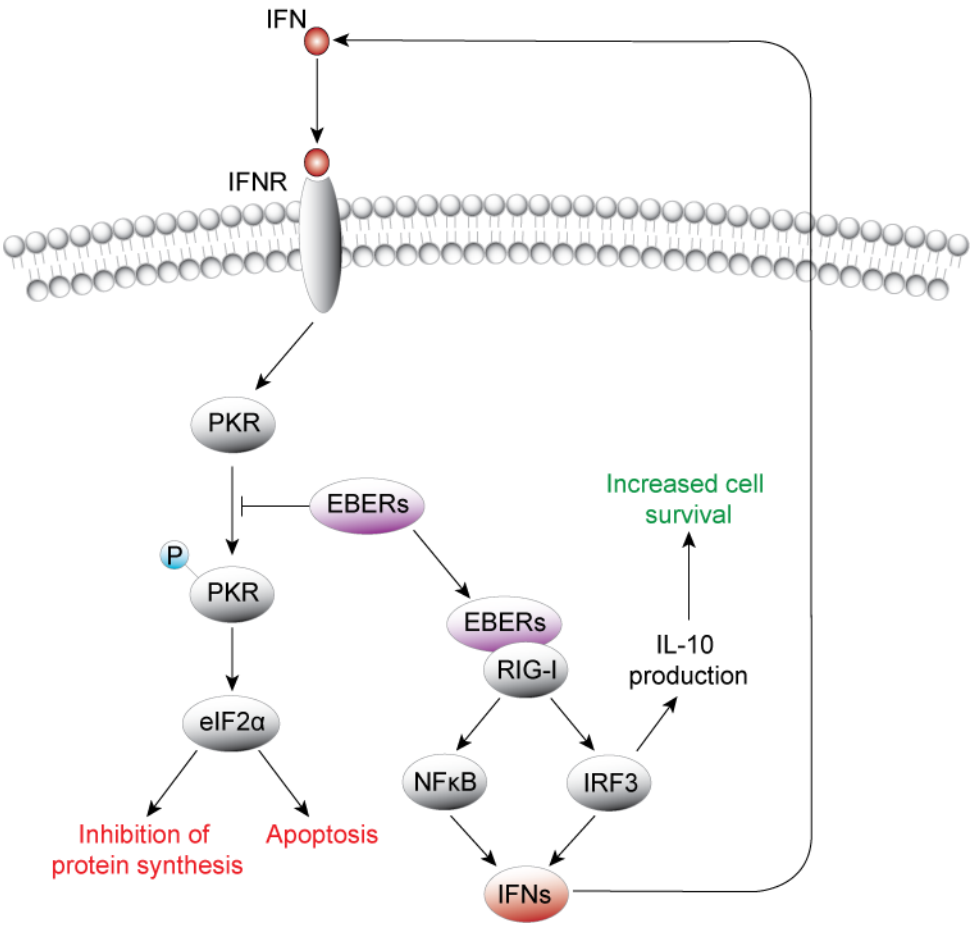


Figure 1.13. This figure is adapted from Samanta et al., 2008 and shows a schematic diagram of the mechanisms of apoptosis protection by EBERs in BL cell lines. Interferon induced apoptosis is blocked through an inhibition of PKR mediated inactivation of eIF2α. In an independent mechanism EBERs induce IL-10 production through binding to RIG-I, while the action of the IFNs produced by this interaction is blocked by the aforementioned effect of EBERs on PKR.

Independent of their interaction with PKR, EBERs have also been shown to increase IL-10 production in BL cells (Kitagawa et al., 2000), which in turn has been implicated in an increase in mitogen-activated protein kinase (MAPK) signalling via increased intracellular ROS (Cerimele et al., 2005). The mechanism of IL-10 induction appears to be through binding of EBERs to the cytosolic viral double-stranded RNA activated protein, retinoic acid-inducible gene 1 protein (RIG-I) (Samanta et al., 2006). Activation of RIG-I leads to IRF3 signalling and downstream activation of IL-10; however both the IRF3 and the NFκB pathway (also activated by RIG-I) lead to an antiviral response through IFN production (Yoneyama et al., 2004). It has been proposed, however, that IFN stimulation through this mechanism is blocked by the aforementioned action of EBERs on PKR (Samanta et al., 2008).

The association of EBERs with increased autocrine growth factor production also seems to extend into non-B cell backgrounds; EBV infection or EBER expression in gastric carcinoma cell lines increases expression of the insulin-like growth factor 1 (IGF-I) (Iwakiri et al., 2003), while EBV Latency II infection or EBER expression in MT-2 T cells increased IL-9 secretion, which was concomitant with shorter doubling times and higher cell saturation density (Yang et al., 2004; Yoshiyama et al., 1995). Thus, although the mechanism is still under debate, there is substantial evidence that EBERs contribute to the EBV-positive phenotype in BL cells.

1.15.3 BARTs

BART transcripts have been observed in all forms of EBV infection (Bell et al., 2006; Brooks et al., 1993; Chen et al., 1992a; Karran et al., 1992; Sadler and Raab-Traub, 1995b; Zhang et al., 2001). Numerous transcripts are generated from the BART region including the mRNAs which encode the RK-BARF0 (Fries et al., 1997; Kienzle et al., 1998; Kusano and Raab-Traub, 2001), A73 (Smith et al., 2000) and RPMS1 proteins (Smith et al., 2000). Little is currently known about the oncogenic potential of the BARTs; however BART derived proteins have been implicated in modulation of several cellular pathways including the highly conserved notch signalling cascade (Kusano and Raab-Traub, 2001; Thornburg et al., 2004; Smith et al., 2000). It has been reported that the RK-BARF0 protein binds to the cytosolic domains of the notch receptor (Kusano and Raab-Traub, 2001) and the human I-mfa domain-containing (HIC) protein resulting in their proteasomal degradation (Thornburg et al., 2004).

RK-BARF0 also interacts with epithelin and scramblase, although this does not result in their degradation (Thornburg et al., 2004). Similarly, RPMS1 can act as an antagonist of EBNA-2 or Notch1 transcriptional activation and A73 is believed to interact with RACK1 (Smith et al., 2000). However, despite these potentially interesting interactions, RK-BARF0, A73 and RPMS1 protein have not been detected in EBV-infected human cells (including BL cell lines) and humans have little detectable antibody to these proteins (Al-Mozaini et al., 2009; van Beek et al., 2003). Therefore it appears unlikely that BARTs contribute to apoptosis resistance in BL cells.

1.15.4 EBV encoded microRNAs

EBV is currently believed to encode 42 miRs from 2 distinct regions of the genome (Barth et al., 2008; Cosmopoulos et al., 2008; Cai et al., 2006; Grundhoff et al., 2006; Pfeffer et al., 2004; Zhu et al., 2009). Most of these are yet to be assigned a function, but expression of two miRs already suggests that they could play an important role in EBV-mediated protection from apoptosis. Bioinformatic analysis predicted an interaction between the BHRF1-3 miR and the mRNA which encodes the IFN-inducible T-cell attracting chemokine, CXCL-11/I-TAC. This was subsequently verified by analysis of EBV-positive DLBCL biopsies, indicating a role for miR-BHRF1-3 miRNA during EBV-driven cell proliferation or lytic replication *in vivo* via inhibition of the interferon response (Xia et al., 2008). Perhaps more relevant for this thesis, the miR-BART5 miR was found to target the 3' untranslated region (UTR) of the cellular mRNA transcript encoding the pro-apoptotic Bcl-2 family member protein, Puma (Choy et al., 2008). In NPC cells, binding of miR-BART5 to Puma mRNA resulted in down-regulation of Puma protein and an increased resistance to adriamycin and etoposide induced apoptosis. Investigation into expression of these miRs in BL cells may provide insights into their possible role in EBV-mediated protection from apoptosis or the expression of novel miRs may provide a key to the EBV-positive phenotype.

1.16 Aims and objectives

It appears from all the data described above that the principle contribution of EBV in the pathogenesis of BL is to counteract the extreme sensitivity to apoptosis that is an inevitable accompaniment of c-myc gene deregulation. An interesting opportunity to investigate this possibility further comes from the

spontaneous loss of the EBV genome from rare BL tumours *in vitro*. However despite a well established link between EBV and apoptosis protection in this system, the mechanism of EBV-mediated protection remains under discussion. In addition, much of the current work has been carried out using a late passage culture of the low incidence sporadic form of BL.

The objective of this thesis is to investigate the role of EBV in the pathogenesis of endemic BL using EBV-loss clones from BL cell lines. In part1 we aimed to generate novel EBV-positive and EBV-loss clones from a panel of EBV-positive Latency I BL cell lines in both early and late passage. We then compared the phenotype of EBV-positive and EBV-loss clones to determine the contribution of EBV to BL growth and apoptosis resistance.

In part2, we used gene expression profiling to investigate cellular gene expression in EBV-positive and EBV-loss clones. We used this data to examine whether BL clones retained a mBL signature and to try and investigate which cellular genes may be responsible for the differences in phenotype between EBV-positive and EBV-loss clones.

The aims of the final part were twofold. First we investigated the effect on EBV-loss clones of infection with recombinant EBV. Next we examined which EBV gene products may be responsible for the increased apoptosis resistance observed in EBV-positive clones. Initially we evaluated the apoptosis protection offered by the EBNA1 protein and the EBER transcripts using EBV derived plasmid vectors. We then analysed expression of the EBV lytic cycle genes and investigated if their expression played a role in apoptosis resistance.

2. Methods

2.1 Cell culture

BL cell lines were grown in BL medium (RPMI1640 medium (Gibco or Sigma) supplemented with 10% v/v selected foetal calf serum (FCS), 6mM glutamine (Gibco) and 8µg/mL gentamycin) at 37˚C, 5% CO_2, in 25cm^2 flasks. Cells were passaged twice weekly by replacement of 80% of BL medium/cell mix with fresh BL medium. Sensitive BL cell lines were grown in αTG medium (normal BL medium supplemented with 1mM pyruvate (Gibco), 50µM alpha thioglycerol (αTG) (Sigma) and 20nM bathocupronine disulfonic acid (BCS) (Sigma), diluted in sterile 1x Phosphate buffered saline (PBS) (Oxoid)).

Human fibroblast cells were grown in DME/HEPES medium (Gibco) supplemented with 10% v/v FCS, 6mM glutamine (Gibco) and 8µg/mL gentamycin at 37˚C, 5% CO_2, in 75cm^2 flasks. In order to passage fibroblasts, medium was removed and cells washed in 1x PBS to remove serum. Adherent cells were dislodged by the addition of 5mL 0.5% w/v trypsin (Gibco) and 5 minutes incubation at 37˚C. Any remaining cells were washed off with warm medium and cells were pelleted by centrifugation at 1,400 RPM for 4 minutes in a large benchtop centrifuge before resuspension in supplemented DME/HEPES medium.

2.1.1 Cryopreservation of cells

Approximately 5x10^6 cells were pelleted by centrifugation at 1,400 RPM for 4 minutes and resuspended in 1mL of freezing mix (50% v/v BL medium, 40% v/v FCS and 10% v/v dimethyl sulphoxide (DMSO)). Cells were placed in a Mr. Frosty container, surrounded by a sponge soaked in propan-2-ol and stored for at least 4 hours at -80˚C before being transferred to the vapour stage of a liquid nitrogen freezer at -180˚C for long term storage.

2.1.2 Recovery of cells from liquid nitrogen

Exposure to DMSO was minimised by rapidly thawing cells at 37˚C, transferral to a 20mL universal centrifuge tube and dropwise addition of 10mL αTG BL medium, which had been prewarmed to 37˚C. Once the DMSO had been diluted out, cells were pelleted by centrifugation at 1,400 RPM for 4

minutes, resuspended in αTG BL cell medium and transferred to wells of a 24 well plate containing $2x10^4$ human fibroblast cells to act as a feeder layer. Cell cultures were expanded at 37˚C, 5% CO_2, until they could be transferred to $25cm^2$ flasks.

2.1.3 Single cell cloning

To prevent EBV lytic replication, BL cells were grown for two weeks in BL medium supplemented with 200μM acyclovir (ACV) (Sigma). To create a feeder layer, human fibroblast cells were seeded into wells of round bottom 96 well plates at a concentration of $2x10^3$ cells/well and incubated at 37˚C, 5% CO_2 for 24 hours to allow then to adhere. BL cells in the exponential growth phase were diluted in ACV-supplemented BL medium and seeded on to the human fibroblasts at a concentration of 0.5, 1 and 3 cells per well. Clones were grown at 37˚C, 5% CO_2 and the media replaced once a week until there were enough cells to transfer to a 48 well plate or to harvest for DNA extraction and quantitative DNA-PCR for viral load.

For this study, single cell cloning of BL cell lines was carried out with help from Dr Gemma Kelly. Cloning of Sav-BL, Ava-BL and Rael-BL was carried out exclusively by the author and cloning of Sal-BL, Oku-BL, Awia-BL and Akata-BL was carried out exclusively by Dr Kelly. The remaining cell lines were single cell cloned by both parties.

2.2 Detection of EBER RNA by flow cytometry

The presence of EBERs was detected using a peptide nucleic acid (PNA), fluorescein isothiocyanate (FITC) labelled, fluorescent probe (Dako) which is able to bind small nuclear RNA (snRNA) EBER1 and EBER2 transcripts. In order to avoid ribonuclease activity, which could degrade the EBERs, all solutions were prepared using water pre-treated with 0.1% diethyl pyrocarbonate (DEPC) (Sigma). All work was carried out whilst wearing gloves and using sterile filter tips (Starlab).

Approximately $2x10^6$ cells were washed in 1x PBS, centrifuged at 1,400 RPM for 4 minutes and fixed in 1mL 5% v/v acetic acid in 4% w/v paraformaldehyde in 1x PBS. An aliquot of $1x10^6$ cells was then transferred to a sterile 1.5mL microcentrifuge tube, centrifuged in a microcentrifuge at 6,000 RPM for 5 minutes and the supernatant aspirated and discarded. Cells were permeabilised for 10 minutes at room temperature in 50μL permeabilisation buffer (0.5% Tween 20 in 1x PBS). To increase probe

binding, 100µL DEPC treated water, 75µL formamide and 25µL formamide buffer (100mM NaCl, 50mM Na_2EDTA, 500mM Tris-HCl pH 7.5) were added to the cells. This ensures a formamide and buffer concentration similar to the hybridization buffer that contains the EBER probe. The mixture was vortexed briefly, centrifuged at 6,000 RPM for 5 minutes and the supernatant aspirated and discarded. 50µL EBER PNA probe (Dako) in hybridization buffer (10% w/v dextran sulphate, 10mM NaCl, 30% v/v formamide, 0.1% w/v $Na_4P_2O_7$, 0.2% w/v polyvinylpyrrolidone, 0.2% w/v ficoll, 5mM NaEDTA, 50mM Tris-HCl pH 7.5) was added and probe binding was carried out at 56˚C for 1 hour. 1mL permeabilisation buffer was added and the mixture incubated for a further 10 minutes at 56˚C. Cells were pelleted by centrifugation and unbound probe was washed from cells by 30 minutes incubation at 56˚C in 1mL permeabilisation buffer. Finally, cells were pelleted and resuspended in permeabilisation buffer ready for flow cytometry.

The percentage of EBER positive cells was measured used a Beckman Coulter XL flow cytometer. Fixed cells were gated on forward and side scatter and the EBER expression was detected though the FITC channel. The threshold of detection was set at 2% for EBV negative cells. For each sample, 5,000 cells were counted and the percentage of positively stained cells recorded.

2.3 Work with DNA and RNA

All work using DNA and RNA samples was carried out whilst wearing gloves and using sterile filter tips (Starlab). Unless otherwise stated, DNA and RNA samples were isolated using commercial extraction kits and diluted in DNase and RNase free water (Sigma). Whenever possible RNA samples were kept on ice to reduce degradation.

2.3.1 DNA extraction

DNA was typically extracted from $5x10^6$ cells. Cells were washed in 1x PBS to remove serum and DNA was extracted using the Qiagen DNeasy kit according to the manufacturer's instructions. DNA was eluted in 100µL water (Sigma) and the concentration determined using a Nanodrop (Thermo Scientific) according to the manufacturer's instructions. DNA samples were typically diluted to a concentration of 100ng/µL and stored at -20˚C.

2.3.2 RNA extraction

RNA was typically extracted from $5x10^6$ cells in exponential growth. Cells were washed in 1x PBS to remove serum and RNA was extracted using an RNA Nucleospin kit (Macherey Nagel) according to the manufacturer's instructions. RNA was eluted in 50µL water (Sigma) and the concentration determined using a Nanodrop (Thermo Scientific) according to manufacturer's instructions. Samples were typically diluted to 100ng/µL and stored at -80˚C.

2.3.3 DNase treatment of cDNA samples

The Macherey Nagel RNA Nucleospin kit used to extract RNA from BL samples has a DNase step designed to remove any residual DNA from RNA samples. However we found that in some cases small quantities of contaminating DNA can be found in preparations of RNA. Most of the RT-PCR assays used were unaffected by residual genomic DNA as they are designed with either a primer or probe spanning an exon-exon splice boundary. This ensures that only cDNA transcripts are amplified during the PCR reaction. However some of the QPCR assays are designed against unspliced EBV transcripts. These include assays for the EBER transcripts and all of the EBV lytic cycle transcripts, except F-U, BZLF1 and BHRF1. For quantitative RT-PCR analysis of these transcripts, aliquots of RNA were first treated with DNase I. 1µg of RNA was treated with the Ambion DNA-*free* DNase kit according to the manufacturer's instructions. DNase I treated RNA was then reversed transcribed to cDNA as normal.

2.3.4 Reverse Transcription

Between 400ng and 1µg RNA was transferred to a thin walled, 0.5mL PCR tube and heated to 90˚C for 3 minutes to denature the secondary structure before being plunged into ice. Reverse transcription of denatured RNA was carried out in a 20µL reaction containing 1x avian myeloblastosis virus reverse transcriptase (AMV-RT) reaction buffer (50mM Tris-HCL pH 8.5, 30mM potassium chloride (KCl), 8mM magnesium chloride ($MgCl_2$), 1mM dithiothreithol (DTT)) (Roche), 200µM each of dATP, dCTP, dGTP and dTTP (Roche), 1µM of the 3' specific primer (Alta Bioscience) (Table 3 and Table 4) and 5 units of AMV-RT (Roche). The reaction was incubated at 42˚C for 1 hour, followed by 5 minutes at 90˚C to

inactivate the AMV-RT and aliquots of cDNA were stored at -20˚C until required. In some cases gene specific primers were replaced with 100ng random primers (Promega) and the reaction mixture was incubated at room temperature for 10 minutes before incubation at 42˚C.

2.4 TaqMan quantitative polymerase chain reaction (QPCR)

2.4.1 Principle of real-time PCR using TaqMan probes

Despite its high sensitivity, conventional PCR is unable to quantify the initial amount of starting material in a sample because only information on the end point of the reaction can be obtained. Quantitative PCR, on the other hand, can be used to make measurements during the exponential phase of amplification before any components of the reaction are depleted and the rate of amplification diminished. This sensitive method can be used to quantify both DNA and RNA and is able to discriminate between as little as 2-fold differences in the amount of starting material.

The ABI Prism 7700 Sequence Detection System or 7500 Real-Time PCR System (Applied Biosystems) were used to continuously measure the accumulation of PCR products using dual labelled TaqMan probes (Heid et al., 1996). TaqMan probes are labelled at the 5' end with a fluorescent reporter (either FAM or VIC) and at the 3' end with a fluorescence quencher (TAMRA or BHQ). While both reporter and quencher remain attached to the same oligonucleotide probe the quencher prevents emission of fluorescence from the reporter. During the PCR reaction, primer extension leads to cleavage of the probe by the exonuclease activity of the DNA polymerase. This leads to spatial separation of the quencher and reporter and an increase in the fluorescence signal. Figure 2.1 shows a schematic representation of the principle of this method.

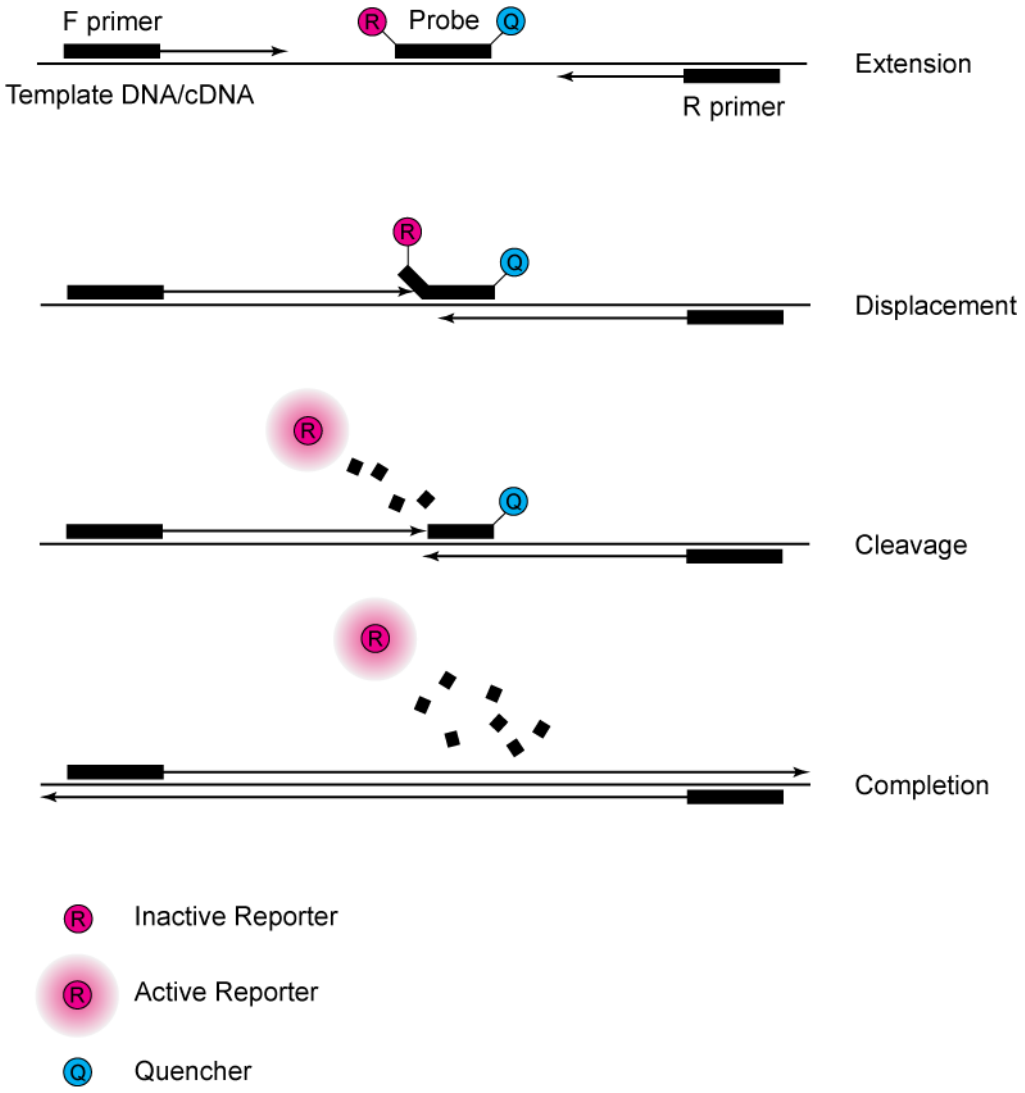


Figure 2.1. Schematic representation of the basic principles of TaqMan quantitative PCR. The primers and probe are designed to bind and amplify the region of interest on the template DNA or cDNA. The probe has a fluorescent reporter at the 5' end and a fluorescence quencher at the 3' end. While the reporter and quencher are in close proximity there is minimal fluorescence, but during the PCR reaction extension of the 5' primer leads to displacement and cleavage of the reporter leading to an increase in fluorescence signal.

Another advantage of quantitative PCR is the ability to detect several different PCR products by multiplex PCR using probes labelled with different fluorescent dyes. This allows, for example, for the measurement of a gene of interest and a cellular control gene within the same reaction.

Fluorescence was measured continuously through 40 cycles of PCR and the change in fluorescence intensity (ΔRn) plotted against the number of cycles for each sample to generate an amplification plot. A threshold intensity is set during the exponential phase of the reaction before reagents become limiting. The number of cycles required for the fluorescence intensity of a sample to cross the threshold determines its Ct value, which is inversely proportional to the initial amount of starting material. If standards of known or relative concentration are included, these can be used to construct a calibration curve of $\log_{10}$ of the initial concentration against Ct. Using the calibration curve the amount of starting material in a sample can be determined from its Ct value. Normalisation of the value generated for a gene of interest against a cellular control gene can be used to compensate for any variation in sample input or quality. For example when measuring EBV viral load by quantitative DNA-PCR, values were normalised against the MHC class I component Beta-2 microglobulin (B2m). Expression of mRNA transcripts measured by quantitative RT-PCR was normalised against the metabolic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.4.2 Primer and probe design

EBV genome load was determined by quantitative DNA-PCR using a probe specific for the EBV polymerase (pol) BALF5 gene. This probe was labelled with FAM at the 5' end and with TAMRA at the 3' end (Eurogentec) (Gallagher et al., 1999). The probe used to detect the B2m control gene was labelled with VIC at the 5' end and with BHQ at the 3' end (Eurogentec).

QRT-PCR assays for the EBV latent transcripts (with the exception of EBER1 and EBER2) and for the lytic BZLF1 and FU transcripts are described in (Bell et al., 2006). Assays for the EBERs and the remaining EBV lytic cycle transcripts were subsequently developed in our lab by Dr Andrew Bell. Probes were labelled at the 5' end with FAM and at the 3' end with TAMRA (Eurogentec). The Probe used to detect the cellular control gene GAPDH was labelled at the 5' end with VIC and with BHQ at the 3' end (Applied Biosystems). The expression of selected cellular genes was measured using QPCR assays purchased from Applied Biosystems. Full lists of EBV transcripts investigated, along

with their primer and probe combinations, are shown in Tables 3-5. EBV coordinates are based on the revised B95-8 sequence (de Jesus et al., 2003).

2.4.3 DNA-PCR to measure EBV genomes

DNA was extracted from cultured cells using a DNeasy Tissue Extraction Kit (Qiagen) as described in 2.3.1. 25µL reactions were set up containing primer and probe combinations specific for both the EBV polymerase (pol) BALF5 gene and cellular B2m gene (Table 2) in wells of a 96 well Optical Reaction Plate (Applied Biosystems). Each reaction consisted of 5ng DNA, 1x TaqMan Universal PCR Master Mix (Applied Biosystems), 200nM of each EBV pol primer, 200nM of EBV pol probe (Eurogentec), 60nM B2m 5' primer, 80nM B2m 3' primer and 100nM B2m probe (Applied Biosystems). Viral load was quantified using serial dilutions of DNA from the reference BL cell line Namalwa-BL, which is known to contain 2 integrated copies of EBV per cell. The wells were sealed with Microamp Optical Caps (Applied Biosystems) and thermocycling and fluorescence detection was carried out in an ABI Prism 7700 Sequence Detection System or 7500 Real-Time PCR System (Applied Biosystems). The samples were heated to 50˚C for 2 minutes, 95˚C for 10 minutes to activate the Amplitaq Gold, followed by 40 cycles of denaturation at 95˚C for 15 seconds and primer annealing and extension at 60˚C for 1 minute.

Target	Primer	Sequence	Final conc.	B95.8 coords
EBV pol	5' primer	5'-AGTCCTTCTTGGCTAGTCTGTTGAC-3'	200nM	154828-154804
	3' primer	5'-CTTTGGCGCGGATCCTC-3'	200nM	154738-154754
	Probe	5'(FAM)CATCAAGAAGCTGCTGGCGGCCT(TAMRA)-3'	200nM	154779-154757
B2m	5' primer	5'-CTTTGGCGCGGATCCTC-3'	60nM	-
	3' primer	5'-AGTCCTTCTTGGCTAGTCTGTTGAC-3'	80nM	-
	Probe	5'-(VIC)CATCAAGAAGCTGCTGGCGGCC(BHQ)-3'	100nM	-

Table 2. Oligonucleotide primer and probe sequences and combinations used for QPCR analysis of EBV DNA load.

2.4.4 RT-PCR

2.4.4.1 Reverse transcription of RNA to cDNA

cDNA was transcribed from 400ng total RNA in a 20µL reaction volume as described in 2.3.4. cDNA corresponding to latent EBV transcripts was primed using a mix of 3' primers specific for EBV latent genes plus cellular GAPDH (Table 3). Lytic EBV transcripts, the EBERs and selected cellular genes were reverse transcribed using random primers.

2.4.4.2 RT-PCR amplification of cDNA

A 25µL reaction was set up containing primer and probe combinations specific for both the gene of interest (Table 3 and Table 4) and the cellular control gene GAPDH, in wells of a 96 well Optical Reaction Plate (Applied Biosystems). Each reaction consisted of 25ng cDNA, 1x Universal PCR Master Mix (Applied Biosystems), the indicated concentrations of primer and probes (Table 3 and Table 4) and 1x GAPDH pre-developed assay (PDAR; Applied Biosystems cat. no. 402869). The wells were sealed with Microamp Optical Caps (Applied Biosystems) and thermocycling and fluorescence detection were carried out in an ABI Prism 7700 Sequence Detection System or 7500 Real-Time PCR System (Applied Biosystems). The samples were heated to 50˚C for 2 minutes, 95˚C for 10 minutes to activate the Amplitaq Gold, followed by 40 cycles of denaturation at 95˚C for 15 seconds and primer annealing and extension at 60˚C for 1 minute.

A standard cell line was selected for each assay based on known physiological high level expression of the transcript of interest (Table 3 and Table 4). Expression of latent Q-U-K transcripts was measured relative to the Latency I BL cell line Rael-BL; Cp initiated transcripts were measured relative to the Cp using LCL, Oku-LCL and EBNA2, LMP1, EBER and Wp transcripts were measured relative to the Wp using LCL, X50-7. Expression of EBV lytic genes was measured relative to 2 lytically active LCL cell lines (Sal-LCL and IMI05-LCL) where around 2% of cells are known to be in lytic cycle. Serial dilutions of the appropriate standard cell line were analyzed alongside unknown samples and used to generate a calibration curve. The relative expression of a gene of interest was then calculated in unknown samples by comparison to the calibration curve.

Transcript (control cell line)	cDNA 3' primer (B95.8 coordinates)	Primer/probe combinations	Final conc	Oligonucleotide sequence (B95.8 coordinates)
Cp-initiated (Oku LCL)	5'-CCTAGGCCCTGAAGG-3' (17631-17626/14832-14824)	5' primer (C_1C_2) 3' primer (W_1W_2) Probe (W_1)	1µM 1µM 200nM	5'-AATCATCTAAACCGACTGAAGAAACAG-3' (11467-11479/11626-11639) 5'-GAGGGGACCCTCTGGCC-3' (14709-14701/14619-14612) 5'(FAM)ACCGCCGTGAAGGCCCTGGACCAAC(TAMRA)-3' (14564-14588)
Wp-initiated (X50-7)		5' primer (W_0) 3' primer (W_1W_2) Probe (W_1)	100nM 100nM 200nM	5'-CGCCAGGAGTCCACACAAAT-3' (14391-14410) 5'-GAGGGGACCCTCTGGCC-3' (14709-14701/14619-14612) 5'(FAM)ACCGCCGTGAAGGCCCTGGACCAAC(TAMRA)-3' (14564-14588)
Q-U-K (Rael)	5'-GTACCTGGCCCCTCGTCA-3' (107972-107955)	5' primer (Q) 3' primer (UK) Probe (U)	1µM 1µM 200nM	5'-GTGCGCTACCGGATGGC-3' (62440-62456) 5'-CATGATTCACACTTAAAGGAGACGG-3' (107952-107942/67649-67636) 5'-(FAM)TCCTCTGGAGCCTGACCTGTGATCG(TAMRA)-3'
EBNA2 (X50-7)	5'-GCAATATAGAATGTAGGCAT-3' (48523–48504)	5' primer, type 1 (Y2/YH) 5' primer, type 2 (Y2/YH) 3' primer (YH) Probe (YH)	300nM 300nM 300nM 200nM	5'-GCTTAGCCAGTAACCCAGCACT-3' (47990-47999/48386-48397) 5'-GCTTAGCCAGTAACTCAGCGCT-3' (47990-47999/48386-48397) 5'-TGCTTAGAAGGTTGTTGGCATG-3' (48469-48448) 5'-(FAM)CCCAACCACAGGTTCAGGCAAAACTTT(TAMRA)-3' (48441-48415)
LMP1 (X50-7)	5'TAGATAGAGAGCAATAATGAGCAG-3' (168841-168864)	5' primer (exon 2) 3' primer (exon 3) Probe (exon 2-3)	300nM 300nM 200nM	5'-GCACGGACAGGCATTGTTC-3' (168893-168912) 5'-AAGGCCAAAAGCTGCCAGAT-3' (168893-168912) 5'(FAM)TCCAGATACCTAAGACAAGTAAGCACCCGAAGAT(TAMRA)-3' (168951–168965/169042–169060)

Table 3. Oligonucleotide primer and probe sequences and combinations used for quantitative RT-PCR analysis of EBV latent transcripts (part1)

Transcript (control cell line)	cDNA 3' primer	Primer/probe combinations	Final conc	Oligonucleotide sequence (B95.8 coordinatess)
EBER1	Primed with random primers	5' primer	300nM	5'-TGCTAGGGAGGAGACGTGTGT-3' (6655-6676)
(X50-7)		3' primer	300nM	5'-TGACCGAAGACGGCAGAAAG-3' (6750-6770)
		Probe	200nM	5'-(FAM) AGACAACCACAGACACCGTCCTCACCA(TAMRA)-3' (6700-6726)
EBER2	Primed with random primers	5' primer	300nM	5'-AACGCTCAGTGCGGTGCTA-3' (6995-7015)
(X50-7)		3' primer	300nM	5'-GAATCCTGACTTGCAAATGCTCTA-3' (7061-7085)
		Probe	200nM	5'-(FAM)CGACCCGAGGTCAAGTCCCGG(TAMRA)-3' (7015-7036)
GAPDH	5'-GATCTCGCTCCTGGAA-3'	Commercial QPCR assay from ABI cat. no. 402869	-	5'-GAAGGTGAAGGTCGGAGTA-3'
			-	5'-GAAGATGGTGATGGGATTTC-3'
			-	5'-(FAM) CAAGCTTCCCGTTCTCAGCC(TAMRA)-3'

Table 3 (continued). Oligonucleotide primer and probe sequences and combinations used for quantitative RT-PCR analysis of EBV latent transcripts (part2)

Transcript (control cell line)	cDNA 3' primer (B95.8 coordinates)	Primer/probe combinations	Final conc	Oligonucleotide sequence (B95.8 coordinates)
Fp-initiated (Sal LCL)	5'-CTTAAAGGAGACGGCC-3' (107972–107955)	5' primer (F) 3' primer (U) Probe (U)	1µM 1µM 200nM	5'-GGGTGAGGCCACGCTTT-3' (62387–62403) 5'-CAGGTCTACTGGCGGTCTATGAT-3' (67614–67592) 5'(FAM)-TCCTCTGGAGCCTGACCTGTGATCG-(TAMRA)3' (67563–67587)
BZLF1 (IMI05 LCL)	5'-GCAGCCACCTCACG-3' (102334–102341/102426–102431)	5' primer (exon 1) 3' primer (exon 2) Probe (exon 1/2)	300nM 300nM 200nM	5'-ACGACGCACACGGAAACC-3' (102688–102671) 5'-CTTGGCCCGGCATTTTCT-3' (102456–102473) 5'(FAM)-GCATTCCTCCAGCGATTCTGGCTGTT-(TAMRA)3' (102519–102530/102655–102668)
BRLF1 (IMI05 LCL)	Primed with random primers	5' primer 3' primer Probe	300nM 300nM 200nM	5'-TTGGGCCATTCTCCGAAAC-3' (106747-106765) 5'-TATAGGGCACGCGATGGAA-3' (106683-106702) 5'(FAM)-AGACGGGCTGAGAATGCCGGC-(TAMRA)3' (106714-106735)
BMLF1 (IMI05 LCL)	Primed with random primers	5' primer 3' primer Probe	300nM 300nM 200nM	5'-CCCGAACTAGCAGCATTTCCT-3' (84246-84266) 5'-GACCGCTTCGAGTTCCAGAA-3' (84093-84112) 5'(FAM)-AACGAGGATCCCGCAGAGAGCCA-(TAMRA)3' (84121-84144)
BHRF1 (IMI05 LCL)	Primed with random primers	5' primer 3' primer Probe	300nM 300nM 200nM	5'-GGCTTACCTCGGTTCCCTCTTA-3' (53883-53895/54335-54335) 5'-TCCCGTATACACAGGGCTAACAGT-3' (54400-54424) 5'(FAM)-TGCCAGATCTTGTAGAGCAAGGATGGCCTATT-(TAMRA)3' (543485-543515)
BNLF2A (IMI05 LCL)	Primed with random primers	5' primer 3' primer Probe	300nM 300nM 200nM	5'-TGGAGCGTGCTTTGCTAGAG-3' (167940-167960) 5'-GGCCTGGTCTCCGTAGAAGAG-3' (167890-167911) 5'(FAM)-CCTCTGCCTGCGGCCTGCC-(TAMRA)3' (167914-167933)

Table 4. Oligonucleotide primer and probe sequences and combinations used for quantitative RT-PCR analysis of EBV lytic transcripts (part1)

Transcript (control cell line)	cDNA 3' primer	Primer/probe combinations	Final conc	Oligonucleotide sequence (B95.8 coordinates)
BALF1	Primed with random primers	5' primer	300nM	5'-GGGCAAAGACACGCACGTA-3' (165793-165812)
(IMI05 LCL)		3' primer	300nM	5'-GCCGCGACCAGTAGTCGTA-3' (165732-165751)
		Probe	200nM	5'(FAM)-CATCATCAGCGTCCTGCGCGC-(TAMRA)3' (165767-165788)
BALF4	Primed with random primers	5' primer	300nM	5'-CAAGCTTTCCTTTCCGAGTCT-3' (161742-161763)
(IMI05 LCL)		3' primer	300nM	5'-ACACTGGATGTACGAGGAGAA-3' (161689-16170)
		Probe	200nM	5'(FAM)-TCCAGCCACGGCGACCTGTTC-(TAMRA)3' (161713-161734)
vIL10	Primed with random primers	5' primer	300nM	5'-CAGTGCCTGGTGCTGCTTTA-3' (9703-9724)
(IMI05 LCL)		3' primer	300nM	5'-AGGCATCTCTTAGGTCCCTCAAC-3' (9774-9797)
		Probe	200nM	5'-(FAM)TTGTCACATTGGTCTGTACCTCCACACTCA(TAMRA)-3' (9731-9762)
BVRF2	Primed with random primers	5' primer	300nM	5'-CCACGGCAGTCTACGGTACA-3' (148379-148399)
(IMI05 LCL)		3' primer	300nM	5'-GCGGCATTGGCGTCAT-3' (148461-148479)
		Probe	200nM	5'(FAM)-ACCTTGCGTGGGTCCTGAAGCACTT-(TAMRA)3' (148400-148425)
gp350	Primed with random primers	5' primer	300nM	5'-AGAATCTGGGCTGGGACGTT-3' (92305-92325)
(IMI05 LCL)		3' primer	300nM	5'-ACATGGAGCCCGGACAAGT-3' (92486-92505)
		Probe	200nM	5'(FAM)-AGCCCACCACAGATTACGGCGGT-(TAMRA)3' (92459-92482)

Table 4 (continued). Oligonucleotide primer and probe sequences and combinations used for quantitative RT-PCR analysis of EBV lytic transcripts (part2)

2.5 Cell death, cell growth and cell survival assays

2.5.1 Cell death sensitivity assay

Sensitivity to cell death was determined using a flow cytometric assay that was able to differentiate between live, apoptotic and necrotic cells. 3 days prior to induction of cell death, cells were routinely counted and resuspended to a density of $3x10^5$ cells/mL in $25cm^2$ flasks. This ensured equal confluency in each cell line at the start of the experiment. To induce apoptosis, cells were transferred to a 20mL universal centrifuge tube, pelleted at 1,400 RPM for 4 minutes and resuspended at a concentration of $3x10^5$ cells/mL in fresh BL medium. Aliquots of 100µL ($3x10^4$ cells) were transferred to wells of a flat bottomed 96 well plate (3 wells per condition), before the addition of 100µL 2x apoptosis inducing agent or normal media control. Cell death was induced at 37˚C, 5% CO_2, for 48-72 hours, depending on the drug used.

Live, apoptotic and necrotic cells were detected by dual staining with the fluorescent DNA binding dyes, Syto 16 and propidium iodide (PI). Cells were pelleted into flow cytometry tubes, resuspended in 500µL warm saline and incubated for 1 hour at room temperature with 10µM Syto 16 (Molecular Probes). PI was added to a final concentration of 25µg/mL and cells were analysed immediately by flow cytometry. As shown in Figure 2.2, flow cytometric analysis was used to generate two-dimensional dot plots of Syto 16 (y-axis) versus PI staining (x-axis). Syto 16 is actively pumped into viable cells and PI preferentially enters cells with compromised cell membranes, thus staining necrotic cells. This method allows the discrimination of live cells (Syto 16 positive, PI negative), apoptotic cells (Syto 16 negative, PI negative) and necrotic cells (Syto 16 negative, PI positive).

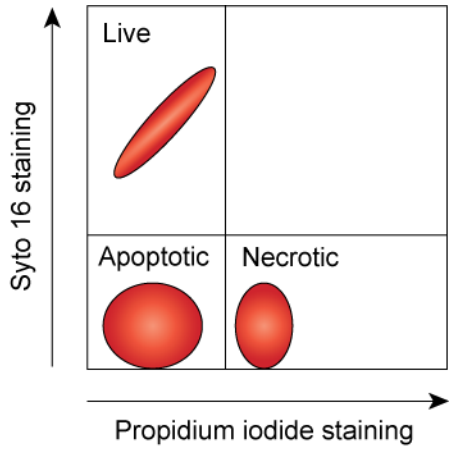


Figure 2.2. Schematic representation of a 2 dimensional dot plot generated by flow cytometric analysis of cells stained with Syto 16 and propidium iodide. This method allows the discrimination of live cells (Syto 16 positive, PI negative), apoptotic cells (Syto 16 negative, PI negative) and necrotic cells (Syto 16 negative, PI positive).

By altering the compensation setting of the flow cytometric analysis it was possible to use PI staining alone to examine total cell death in a sample. This was used to measure cell death in cell lines expressing GFP, which is measured through the same channel as Syto 16. PI staining (y-axis) was plotted against GFP expression (x-axis) (Figure 2.3). This program allowed discrimination of live, GFP-positive cells; live, GFP-negative cells and dead cells. As this program was able to measure cell death in GFP-positive and GFP-negative cells within the same population, we used it to determine the effect on resistance to cell death of plasmid vectors expressing a gene of interest and a GFP marker.

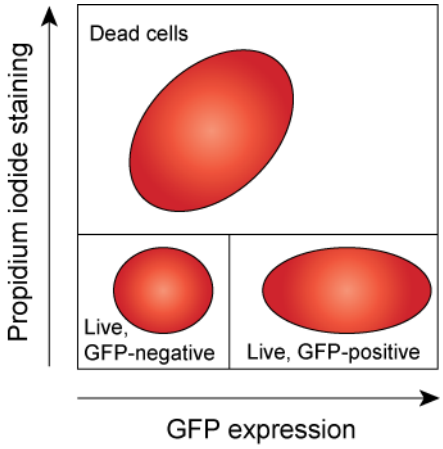


Figure 2.3. Schematic representation of a 2 dimensional dot plot showing propidium iodide staining against GFP expression in a cell line containing GFP expressing cells. This method allows discrimination of live, GFP-positive cells; live, GFP negative cells and dead cells.

2.5.2 Cell growth assays

To determine the number of viable cells/mL in BL cell lines, we used trypan blue staining; 50µL of the culture was mixed with 50µL of trypan blue (Sigma) and the number of negatively stained cells/mL was determined using a haemocytometer. To ensure equal confluency in each cell line at the start of the experiment, cells were pelleted by centrifugation at 1,400 RPM for 4 minutes and resuspended at a concentration of $3x10^5$ viable cells/ml in 10mL fresh BL medium in $25cm^2$ flasks. After 3 days incubation at 37˚C, 5% CO_2, cells were again counted, pelleted and resuspended at a concentration of $3x10^5$ viable cells/mL in 10mL of fresh media in $25cm^2$ flasks. The number of viable cells was determined each subsequent day for the next 6-8 days or until no viable cells remained within the culture.

2.5.3 Cell survival in low serum

The total number of viable cells/mL in each cell line was determined by trypan blue staining as described above. To ensure equal confluency in each cell line at the start of the experiment, cells were pelleted by centrifugation at 1,400 RPM for 4 minutes and resuspended at a concentration of $3x10^5$ viable cells/mL in 10mL of fresh media in $25cm^2$ flasks. After 3 days incubation at 37˚C, 5% CO_2, cells were washed twice in RPMI1640 and allowed to rest in the RPMI1640 overnight to remove any traces of foetal calf serum (FCS). The next day cells were resuspended at a concentration of $3x10^5$ viable cells/mL in 10mL of BL medium containing 1% or 0.1% FCS in $25cm^2$ flasks. The number of viable cells was determined each day for the subsequent 8 days or until no viable cells remained within the culture.

2.5.4 Colony formation assay

Low melting point (LoMP) agarose was dissolved 2% w/v in distilled water, sterilised by autoclaving at 121˚C, 15psi for 15 minutes and while still liquid, transferred to a 37˚C water bath. Warm medium was mixed 1:1 and 5:1 v/v with LoMP agarose to make 1% and 0.33% LoMP agarose solutions. Bases of LoMP agarose were formed by applying 2mL of 1% LoMP agarose to wells of a 6 well plate. Plates were then placed on a cold metal tray for 10 minutes at 4˚C to allow the base layer to solidify.

To test colony forming ability, cells were counted and the appropriate number of cells (between $2.5x10^4$ and $2.5x10^5$ depending on the cell line) were pelleted and resuspended in 2mL of 0.33% agarose. The cell/agarose mixture was pipetted over the solid base layer and plates were placed on a cold metal tray for 10 minutes at 4˚C to fix cells within the agarose matrix. Cells were then incubated at 37˚C, 5% CO_2 and supplemented with 2 drops of BL cell medium weekly until cell colonies appeared. After 4 weeks incubation, the number of colonies per well was recorded and representative fields photographed.

2.6 Protein analysis

2.6.1 Preparation of protein samples

Protein preparations were made from approximately $1x10^7$ cells. Cells were washed in 1x PBS to remove FCS, then pelleted by centrifugation at 1,400 RPM for 4 minutes and lysed in 100µL urea buffer (9M urea, 50mM Tris, pH 7.5). The viscosity of samples was reduced by disruption of genomic DNA by 15 sec sonication using an ultrasonic cell disruptor (Misonix). The protein concentration was determined using a Bio-Rad DC protein assay kit according to the manufacturer's instructions. At least an equal volume of 2x urea gel sample buffer (UGSB) (62.5mM Tris pH6.8, 4% w/v sodium dodecyl sulphate (SDS), 5% B-mercaptoethanol, 0.01% w/v bromophenol blue, 5M urea, 10% glycerol) was used to dilute the samples to a set concentration and diluted samples were denatured at 100˚C for 3 minutes. Protein samples were then separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) or stored at -20˚C.

2.6.2 SDS-PAGE

Depending on the size of protein being investigated and the resolution required, 2 different sizes of SDS-PAGE gels were used. For most samples, small Tris-HCl 4–20%, precast gradient gels (Bio-Rad) were loaded with 20-40µg of protein alongside 10µL of Seeblue plus 2 pre-stained standards (Invitrogen). Electrophoresis was carried out for 90 minutes at 100v in tanks filled with running buffer (25mM Tris-HCl pH8.3, 192mM glycine, 0.1% w/v SDS).

When greater separation of similar sized proteins or resolution of very small or large proteins was required, 2 gel phases were poured into Protean II Bio-Rad gel apparatus. A resolving gel was made up containing 375mM Tris-HCl pH8.8, 0.06% w/v N, N, N' N'-tetramethylethylenediamine (TEMED) (Sigma), 0.1% w/v SDS, 0.003% w/v ammonium persulfate (APS) and 7.5-15% acrylamide (Bio-Rad). The percentage acrylamide used depended on the size of protein that required resolution; 15% acrylamide was used to resolve 10-43kDa proteins, 12.5% acrylamide was used to resolve 12-60kDa proteins, 10% acrylamide was used to resolve 20-80kDa proteins and 7.5% acrylamide was used to resolve 36-94kDa proteins. 200µL of water-saturated butan-2-ol was pipetted on to the gel to give a uniform surface and the mixture was left to completely polymerize for 45 minutes.

The butan-2-ol was washed off and a second stacking gel consisting of 3% acrylamide, 125mM Tris-HCl pH6.8, 0.06% w/v TEMED, 0.1% w/v SDS and 0.15% APS was pipetted on top of the resolving gel and gel-forming combs inserted. After 30 minutes the gel combs were removed and the wells washed out with running buffer. 50-100µg of protein was loaded into the wells of the stacking gel alongside 20µL Seeblue plus 2 pre-stained standards (Invitrogen). Electrophoresis was carried out at 40mA for 20 minutes to run protein samples into the stacking gel, then overnight at 11mA to separate proteins through the resolving gel. To prevent overheating, a water coolant supply at 8˚C was connected to the apparatus.

2.6.3 Western blotting

Once the dye front reached the end of the gel, samples were blotted on to a nitrocellulose membrane in an appropriate sized blotting cassette. On to the cathode side of the blotting cassette was laid a sponge and sheet of 3MM Whatman filter paper pre-soaked in blotting buffer (25mM Tris-HCl pH8.3, 192mM glycine, 20% v/v methanol). This was followed by the nitrocellulose membrane and the gel. On to the gel was placed another sheet of pre-soaked 3MM Whatman filter paper and another sponge. The blotting cassette was placed into a Trans-Blot tank (Bio-Rad) filled with blotting buffer. Large gels were attached to a water coolant supply at 8˚C to prevent overheating and proteins transferred from the gel to the membrane at 85v for 4.5 hours. Small gels were cooled with an ice block and proteins transferred for 90 minutes at 100v. Blotting cassettes were then disassembled, the membrane rinsed briefly in distilled water and incubated in Ponceau S stain (1% Ponceau S w/v (Sigma) in 3% v/v trichloroacetic acid (TCA)) for 5 minutes to visualise protein binding. Membranes were rinsed in distilled water and washed twice for 20 minutes in 1x PBS with 0.1% v/v Tween20 (Sigma) (PBS Tween) to remove the Ponceau S. Non-specific antibody binding was prevented by incubation of the membrane for 1 hour in blocking agent (5% w/v skimmed milk powder in 1x PBS, 0.1% Tween20), with shaking, at room temperature. Membranes were sealed in plastic bags with 10-20mL primary antibody diluted in blocking agent and incubated with shaking either overnight at 4˚C or for 1 hour at room temperature depending on the antibody used (Table 5).

Following incubation the primary antibody was drained off (antibodies were stored at -20˚C and reused several times) and the membrane washed twice for 10 minutes in PBS Tween. The membrane was

then sealed in a bag with the appropriate peroxidase-conjugated secondary antibody (diluted 1 in 1000 or 1 in 2000 in blocking agent) and incubated with shaking at room temperature for 1 hour. The secondary antibody was then discarded and the membrane washed 6 times over 2 hours in PBS Tween.

Enhanced chemiluminescence (ECL) was used to visualize antibody bound proteins. The membrane was incubated with shaking for 1 minute in 10mL each of the ECL reagents A and B (Amersham), before being wrapped in Saranwrap and placed in an autoradiography cassette. Kodak X-Omat AR film was exposed to the membrane for between 1 second and 30 minutes depending on the strength of the luminescence signal. The ECL reagents were removed by washing for 15 minutes in PBS Tween and membranes were stored at 4˚C in Saranwrap to prevent drying out or incubated in blocking agent and re-probed with primary antibody specific against another protein with a different molecular weight.

Target	Antibody	Origin	Dilution	Incubation
EBNA1	1H4	Rat (monoclonal)	1 in 50	O/n 4˚C
EBNA1	A.Mo.	Human (polyclonal)	1 in 100	O/n 4˚C
EBNA2	PE2	Mouse (monoclonal)	1 in 50	O/n 4˚C
LMP1	CS1-4	Mouse (monoclonal)	1 in 20	O/n 4˚C
Tcl-1	MBL K0028-1	Mouse (monoclonal)	1 in 1000	1 hour RT
Bcl-2	Santa Cruz sc-7382	Mouse (monoclonal)	1 in 200	O/n 4˚C
Bcl-XL	Santa Cruz sc-8392	Mouse (monoclonal)	1 in 200	O/n 4˚C
Mcl-1	Santa Cruz sc-12756	Mouse (monoclonal)	1 in 200	O/n 4˚C
Bim (EL,L and S)	Stressgen AAP-330	Rabbit (polyclonal)	1 in 1000	O/n 4˚C
Bax	Santa Cruz sc-493	Rabbit (polyclonal)	1 in 200	O/n 4˚C
Bak	Santa Cruz sc-1035	Goat (polyclonal)	1 in 200	O/n 4˚C
ID2	Santa Cruz sc-489	Rabbit (polyclonal)	1 in 200	O/n 4˚C
NMI	Santa Cruz sc-9482	Goat (polyclonal)	1 in 200	O/n 4˚C
TNFAIP3	Santa Cruz sc-32525	Goat (polyclonal)	1 in 200	O/n 4˚C
β Actin	Santa Cruz sc-1615	Goat (polyclonal)	1 in 200	1 hour RT

Table 5. Antibodies used for western blot analysis

2.6.4 EBNA1 Immunofluorescence (IF) staining

$1x10^6$ cells were transferred to an autoclaved 1.5mL microcentrifuge tube, washed in 1x PBS, pelleted at 3,000 RPM for 3 minutes in a microcentrifuge and resuspended in 500µL of 1x PBS. 10µL of cells were spotted on to wells of a glass slide and air dried overnight at room temperature. Cells were fixed by immersion in 4% paraformaldehyde for 10 minutes and allowed to air dry. 30µL of 1% Triton X was applied to each well for 5 minutes to permeabilise the cells. The Triton X was replaced with 30µL of 10% heat inactivated goat serum (HINGS) in 1x PBS and slides were incubated at room temperature in a sealed moist container for 1-1 ½ hours to block non-specific antibody binding. HINGS was replaced with 30µL R4 anti-EBNA1 primary antibody (kindly provided by Lori Frappier) and the slides incubated for a further 1-1 ½ hours at room temperature in a sealed moist container. Slides were then washed 3 times in 1x PBS and incubated for 1-1 ½ hours at room temperature in a sealed moist container with 30µL of Alexa Fluor conjugated anti-rabbit secondary antibody (Molecular Probes). Excess secondary antibody was removed with 3 washes in 1x PBS and a spot of 1,4-Diazobicyclo-(2,2,2)-octane (DABCO) added to preserve fluorescence. Cells were covered with a cover slip and staining was visualised within 3 days using a fluorescence microscope. Fluorescence staining was frequently photographed with a parallel bright field exposure of the same area also recorded.

2.7 Gene expression profiling

2.7.1 Introduction to gene expression profiling

Conventional and quantitative RT-PCR are highly effective methods for measuring the expression of small numbers of genes, but they require some prior expectation that a gene of interest may be differentially expressed. Gene expression profiling, using microarray technology, allows the measurement of thousands of different genes and can be used to identify novel differentially expressed genes between multiple samples. To search for genes that are differentially expressed between EBV-positive and EBV-loss clones and that may explain the observed differences in cell phenotype, we used Affymetrix human genome U133 plus 2.0 arrays. Each array is able to measure the expression of over 47,000 RNA transcripts, which were generated from the online gene databases

Genbank, dbEST, RefSeq and Unigene. Each of the ~30,000 human genes is represented at least once and in many cases several times on the array.

A schematic illustration of the principle of Affymetrix microarray analysis is shown in Figure 2.4. Briefly, a large quantity of highly purified RNA was extracted from cells using TRIzol and single stranded cDNA was transcribed using oligo dT primers. A second strand was then synthesised and the double-stranded cDNA purified. This double-stranded cDNA was used as a template to generate fluorescently labelled cRNA by *in vitro* transcription, which was purified and fragmented before being hybridised to the microarray chip.

The Affymetrix microarray chip consists of a quartz surface on to which short (25 nucleotide) DNA fragments (probes) are chemically synthesised at precise locations. Binding of fluorescently labelled cRNA to a probe can be detected by high resolution scanning and provides a quantitative measurement of gene expression. Each probe is designed to bind a single RNA transcript and has a paired mismatch probe partner in the same region of the chip. The mismatch probe differs from the matched probe by only a single nucleotide in the centre of the 25 nucleotide sequence. This provides an invaluable internal control, as it binds to non specific sequences as effectively as its matched probe partner, allowing spurious signals due to cross hybridization to be eliminated from the gene expression measurement. In total there are 11 matched and mismatched probes (collectively termed a “probe set”) spanning each of the 47,000 transcripts included on the microarray. Thus each chip contains just over a million probes on its surface.

A number of checks are made to determine the quality of cRNA bound to the array. As stated cDNA is primed from the 3' end of mRNA transcripts using oligo dT primers. To avoid any loss of signal due to incomplete cDNA transcription, most probe pairs are located towards the 3' end of the gene. However, a number of specific control genes such as β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have probe sets located at both the 3' and 5' end of the mRNA transcript. The effectiveness of the oligo dT primed reverse transcription was measured by comparing signals from the 5' to those at the 3' ends. Secondly, prokaryotic poly-A transcripts are spiked into samples during first strand synthesis. Detection of these transcripts provides an indication of the effectiveness of cRNA generation which is independent of the quality of sample RNA.

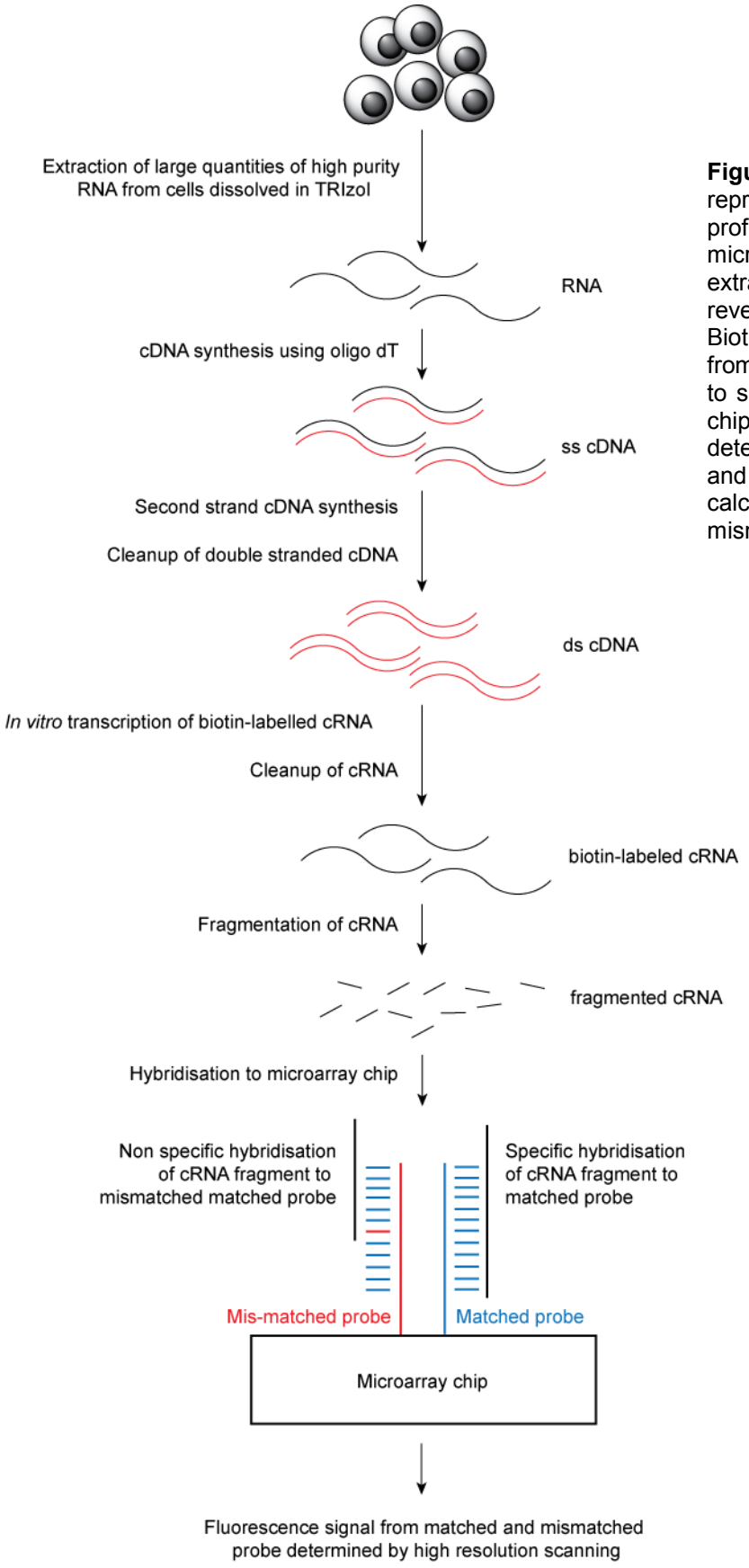


Figure 2.4. Schematic representation of gene expression profiling using the Affymetrix microarray system. RNA is extracted from samples and reverse transcribed into cDNA. Biotin-labelled cRNA is generated from purified cDNA and hybridised to specific probes on a microarray chip. Gene expression is determined by fluorescence signal and non-specific binding is calculated from binding to mismatched probes.

2.7.2 Extraction of RNA for gene expression profiling

To extract the large quantities of high purity RNA required for gene expression profiling, cells were counted, pelleted at 1,400 RPM for 4 minutes and resuspended in TRIzol (Invitrogen) at a concentration of $1x10^7$ cells/mL. RNA is highly stable in TRIzol and aliquots were stored at -80˚C until required. To extract RNA, 1mL of cell/TRIzol mix was transferred to a sterile 1.5mL microcentrifuge tube and centrifuged at 13,000 RPM in a microcentrifuge for 10 minutes to remove any insoluble material. 200µL of chloroform was added and the sample shaken by hand for 15 seconds to mix. After 2 minutes incubation at room temperature the upper aqueous phase containing the RNA was transferred to a new 1.5mL sterile microcentrifuge tube. Another 500µL of chloroform was added, mixed by hand, incubated for 2 minutes at room temperature and centrifuged at 13,000 RPM for 15 minutes at 4˚C. The aqueous layer was again transferred to a new sterile 1.5mL centrifuge tube and 500µL of propan-2-ol added to precipitate the RNA. The mixture was vortexed and incubated at room temperature for 10 minutes. RNA was pelleted by centrifugation at 13,000 RPM for 15 minutes at 4˚C and washed with 1mL 75% ethanol. The mixture was then centrifuged at 8,000 RPM for 5 minutes at 4˚C and the ethanol aspirated and discarded. The RNA pellet was air dried at room temperature, resuspended in 50µL of DEPC treated water and placed on ice. The RNA concentration was determined using a Nanodrop (Thermo Scientific) according to the manufacturer's instructions and diluted in DEPC treated water to a final concentration of 1.25µg/µL, ready for cDNA synthesis.

2.7.3 First strand cDNA synthesis

Throughout cDNA synthesis, RNA samples and all reagents were kept on ice between incubations to prevent degradation. 8µL of 1.25µg/µL total RNA and 1µL of 100µM $T7(dT)_{24}$ primer (Invitrogen) was transferred to a 0.2mL thin-wall, dome capped tube (Alpha Laboratories) and spiked with 2µL poly-A control (Ambion). The poly-A controls consist of 4 poly-adenylated prokaryotic RNA transcripts, which are detected on the microarray and provide a sensitive indicator of the labelling reaction efficiency, independent from the quality of the starting material.

Samples were mixed by pipetting and incubated at 65˚C for 10 minutes to denature RNA transcripts. 4µL 5x first strand buffer (Invitrogen), 2µL 0.1M dithiothreithol (DTT) (Invitrogen) and 1µL 10mM dNTPs (Invitrogen) were added and the mixture incubated at 42˚C for 2 minutes. 2µL of Superscript II

reverse transcriptase (200U/μL) (Invitrogen) was added and first strand cDNA synthesis carried out at 42˚C for 1 hour.

2.7.4 Second strand cDNA synthesis

Small nicks were introduced into the RNA strand of the RNA:DNA duplex by *E.coli* RNases. These nicks provide the binding and primer extension points for DNA polymerase, which transcribes the second cDNA strand. To the product of each first strand cDNA synthesis was added 91μL of DEPC treated water, 30μL of 5x second strand buffer (Invitrogen), 3μL 10mM dNTPs (Invitrogen), 1μL *E.coli* DNA ligase 10U/μL (Invitrogen), 4μL *E.coli* DNA polymerase I 10U/μL (Invitrogen), and 1μL *E.coli* RNase H 2U/μL (Invitrogen). The mixture was then incubated for 2 hours at 16˚C to allow nicking of RNA transcripts. The second cDNA strand was synthesised by the addition of 2μL of T4 DNA polymerase (Invitrogen), followed by 5 minutes incubation at 16˚C and the reaction was halted by the addition of 10μL 0.5M EDTA (Invitrogen).

2.7.5 Cleanup of double-stranded cDNA

The product of second strand cDNA synthesis was purified using the sample cleanup module (Affymetrix) according to the manufacturer's instructions. Purified cDNA was eluted in 14μL of cDNA elution buffer and placed on ice ready for *in vitro* transcription.

2.7.6 *In vitro* transcription (IVT)

Synthesis of fluorescently labelled cRNA was carried out using the IVT labelling kit (Affymetrix). 14μL of DEPC treated water, 4 μL 10x IVT labelling buffer, 12μL IVT labelling NTP mix and 4μL of IVT labelling enzyme mix were added to 6 μL of purified double-stranded cDNA and the mixture incubated overnight at 37˚C. The T7 RNA Polymerase in the IVT labelling enzyme mix uses the ds cDNA as a template for transcription of cRNA. During IVT, biotinylated pseudouridine nucleotide analogs are incorporated into the cRNA transcripts, fluorescently labelling the cRNA.

2.7.7 Cleanup of cRNA

The product of *in vitro* transcription was purified using the sample cleanup module (Affymetrix) according to the manufacturer's instructions and eluted in 10µL of DEPC treated water. The concentration of purified cRNA was determined using a Nanodrop (Thermo Scientific) according to manufacturer's instructions.

2.7.8 cRNA fragmentation

cRNA fragmentation was carried out in a 50µL reaction volume. DEPC treated water and 10µL 5x fragmentation buffer (Affymetrix) was added to 25µg of cRNA and the mixture was incubated at 94˚C for 35 minutes. Transcripts were fragmented by metal ion-catalyzed hydrolysis as a result of heat and magnesium acetate in the fragmentation buffer. Samples were placed on ice and fragmentation and RNA quality checked by running 1µL of fragmented cRNA on a 1.2% formaldehyde agarose gel alongside a 1µg of total RNA and 500ng of unfragmented cRNA. Fragmented cRNA was then handed to members of the Nasopharyngeal Carcinoma (NPC) group, who hybridized it to the Affymetrix U133 plus 2.0 chip. Hybridised chips were scanned using an Affymetrix GeneChip Scanner 3000 according to the Affymetrix technical manual (www.affymetrix.com/support/technical/manual/expression_manual.affx).

2.7.9 Quality control and normalisation of gene expression data

Quality control and normalisation were carried out by The Institute of Cancer Studies bioinformatician, Dr Wenbin Wei. The scanned chip images were analysed using Affymetrix GeneChip Operating Software (GCOS). The quality of cRNA hybridised to the chip was determined by examining the ratio of signals from probes at the 5' and 3' ends of the control β-actin and GAPDH transcripts. The ratio was found to be within acceptable limits in every sample and the poly-A control transcripts spiked into the samples during first strand synthesis could readily be detected.

Probe intensity normalisation was carried out according to the methods of (Irizarry et al., 2003) and (Bolstad et al., 2003) and performed using the Affymetrix package of the Bioconductor project (http://www.bioconductor.org). While it was expected that there would be differences in the expression

of specific genes it was assumed that the mean signal across every probe on a chip would be a constant across all the samples. This assumption allowed spurious differences in signal intensity resulting from differences in basal chip fluorescence or efficiency of hybridisation to be factored out by applying a scaling factor to the fluorescence signals from each chip.

Background binding for each probe was quantified by examining binding to its mismatched probe partner as described in 2.7.1. The background value generated from each mismatch probe was subtracted from the intensity value for its matched probe partner. Gene expression was calculated by examination of the 11 probe partners which span each transcript.

Across the 14 samples tested, between 25.6 to 31.7% of probes on the arrays were found to have bound fragmented cRNA transcripts. This lies within the acceptable range as large numbers of tissue specific genes will not be expressed in BL samples.

2.7.10 Statistical analysis of gene expression data

Statistical significance of differences in gene expression between EBV-positive and EBV-loss clones was determined using rank product analysis (Breitling et al., 2004; Breitling and Herzyk, 2005). Calculations were carried out using the RankProd package from the Bioconductor project (http://www.bioconductor.org). This method compares the expression of all the genes from sample A to those in sample B and calculates the fold change (FC). The genes are then ranked from greatest FC between sample A and sample B to lowest FC between sample A and sample B. Significance is based on the probability that a gene will appear at a position in the rank over several replicates. In noisy biological experiments, a gene could appear at the top of the rank in a single experiment even if there was no difference between sample A and sample B. However over several replicates the probability that a gene could appear at the top of the rank by chance decreases to the point where it becomes statistically probable there is differential expression of this gene between sample A and sample B.

For example, if a very simple array measured the expression of 10 genes in sample A and the same 10 genes in sample B, then the probability that a gene would appear as the top ranked gene in the list would be 0.1, if the expression values were totally random. Therefore the probability that a gene would be at the top of the rank across 2 replicates would be 0.1x0.1 = 0.01 or 1%. So as the number of

replicates increases the probability that a gene could occupy a particular position within the rank by chance decreases. To identify the greatest number of differentially expressed genes the percentage of false-positives was set to a threshold of 10%. Heat maps of gene expression in EBV-positive and EBV-loss clones were generated using dChip software (http://www.dchip.org).

2.8 Conventional RT-PCR

RT-PCR primers were identified from published reports into CD86 (de Haij et al., 2005), NMI (Banchereau et al., 1999) and TNFAIP3 (Cook et al., 2003) expression. Binding of primers to their relevant human mRNA sequences was tested using the Mac Vector software package and Tm values were calculated using Primer Express software (Applied Biosystems).

To amplify CD86 and TNFAIP3 transcripts, RNA was extracted as described in 2.3.2 and cDNA reverse transcribed using AMV-RT (2.3.4). RT-PCR was carried out in a thin walled PCR tube in a 50µl reaction mix containing 20ng of random primed cDNA, 1x PCR reaction buffer (75mM Tris-HCl pH8.8, 20mM ammonium sulphate, 0.01% Tween20) (ABgene), 1.5mM $MgCl_2$ (ABgene), 200µM each dATP, dCTP, dGTP and dTTP (Roche), 1µM of each gene specific primer (

Table 6) and 5 units of Red Hot Taq polymerase (ABGene). Samples were heated to 94˚C for 5 minutes in an Eppendorf Thermocycler and incubated through 35 cycles of 94˚C for 1 minute to denature the DNA strands, 50-60˚C for 1 minute to allow the primers to anneal to the DNA and 72˚C to allow extension of the product. The annealing temperature and extension time at each stage varied according to the specific primers and the product size of each PCR (

Table 6). The products of the PCR reaction were separated by agarose gel electrophoresis (2.9).

Target	Primer	Sequence	Annealing temperature	Extension time
CD86	5' primer 3' primer	5'-GTATTTTGGCAGGACCAGGA-3' 5'-GCCGCTTCTTCTTCTTCCAT-3'	58°C	2 minutes
NMI	5' primer 3' primer	5'-GGCCAAGCCAGTTCCATTAA-3' 5'-CCACATCTACTTCTCCACCTCC-3'	57˚C	1minutes
TNFAIP3	5' primer 3' primer	5'-AAGCTTGTGGCGCTGAAAAC-3' 5'-GAACACCCAGCCTTTATGCCAT-3'	54°C	1 minute

Table 6. Oligonucleotide primer sequences used for conventional RT-PCR

2.9 Agarose gel electrophoresis

Depending on the predicted DNA product size, a 0.8%-3% w/v agarose gel was prepared. Molecular biology grade agarose gel (Eurogentec) was dissolved in 1x TBE (0.09M Tris-borate, 0.002M EDTA) by heating in a microwave oven. Molten agarose was poured into a gel tray, a comb inserted to form wells and the gel allowed to solidify. DNA samples were mixed with 5x loading buffer (0.25% w/v xylene cyanol, 0.25% w/v bromophenol blue, 30% v/v glycerol in 1x TBE) and loaded into the wells alongside a 1kb DNA ladder (Invitrogen) to allow determination of product size. DNA fragments were separated by gel electrophoresis at 90-200v and then stained in 1x TBE buffer containing 0.5mg/mL ethidium bromide. DNA was then visualized and photographed on a UV transilluminator.

2.10 Gel extraction

DNA was separated by gel electrophoresis (above) and the gel stained with ethidium bromide. DNA fragments were visualised by UV light on a transilluminator and bands of the correct size were excised with a scalpel and transferred to an autoclaved 1.5mL microcentrifuge tube. DNA was extracted using a gel extraction kit (Qiagen) according to the manufacturer's instructions, eluted in 50uL of elution buffer and stored at -20˚C until required.

2.11 Digestion of DNA with restriction endonucleases

DNA samples from PCR reactions or plasmid preparations were digested by addition of 10x restriction endonuclease buffer (New England Biolabs or Roche), 1μL of the appropriate restriction endonuclease, 10x BSA solution (if specified for optimal enzyme activity) and water (Sigma). Typically a final volume of 20μL was used and the amount of restriction endonuclease added never exceeded 10% of the total reaction volume. Reactions were incubated at 37˚C or 50˚C (depending on the restriction endonuclease used) for 2-14 hours and an aliquot separated by gel electrophoresis (2.9). Digested DNA products required for DNA ligation were either purified by gel extraction or by phenol chloroform purification and sodium acetate precipitation (below).

2.12 Phenol chloroform purification of DNA

Phenol chloroform purification and sodium acetate precipitation were used to purify and concentrate the products of conventional PCR or DNA digested with restriction endonucleases. DNA samples were made up to a volume of 200µL with water (Sigma). 200µL of phenol chloroform (Sigma) was added and samples mixed thoroughly by vortexing. The aqueous and phenol phases were separated by centrifugation at 13,000 RPM for 2 minutes and the upper aqueous phase (containing the DNA) transferred to an autoclaved 1.5mL microcentrifuge tube. DNA was precipitated by the addition of 0.1x volume 3M sodium acetate and 2.5x volume 100% ethanol. Samples were vortexed and placed at -20˚C for at least 1 hour, then centrifuged at 13,000 RPM for 20 minutes at 4˚C to pellet the precipitated DNA. Samples were washed with 200µL 70% ethanol, allowed to air dry and resuspended in 10µL water (Sigma).

2.13 Ligation of restriction endonuclease digested DNA

Ligation reactions were used to insert DNA fragments into suitable plasmid vectors. The insert and vector were digested with the appropriate restriction endonucleases. The insert DNA was routinely run out on an agarose gel and purified using gel extraction. Following digestion, vectors were capped to prevent self-ligation by addition of 1µL of alkaline phosphatase (Roche) and incubation at 37˚C for 1 hour. Capped vector DNA was then isolated by phenol chloroform purification as described above and an aliquot of both vector and insert were run on an agarose gel to check their relative concentrations. Inserts were then ligated into plasmid vectors using the Rapid Ligation kit (Roche) according to the manufacturer's instructions and the product used to transform DH5α *E.coli* (2.14.2).

2.14 Bacteriology

Plasmid vectors were grown in DH5α bacterial cells. Small scale DNA preparation was used to screen colonies containing recombinant plasmids, while large scale preparations were used to make large amounts of high purity DNA suitable for transfection into mammalian cells.

2.14.1 Bacterial growth medium

Liquid bacterial cultures were grown in Lennox Broth (L-Broth) medium. 2.5% w/v L-broth (Fisher Scientific) was dissolved in 400mL distilled water and sterilized before use by autoclaving at 121˚C, 15psi for 15 minutes.

Bacterial colonies were cultivated on L-Broth Agar (L-agar) plates. 1.5% w/v of agar (Fisher Scientific) was dissolved in 400mL deionised water and sterilized by autoclaving at 121˚C, 15psi for 15 minutes. The L-agar was remelted by microwaving, cooled to around 50˚C and ampicillin added to a final concentration of 100µg/mL. Agar was poured into Petri dishes, left to solidify at room temperature and stored at 4˚C for up to a week. Before use, plates were dried thoroughly at 37˚C.

SOB medium was used to cultivate competent DH5α E.coli. SOB contained 2% w/v tryptone (DIFCO), 0.5% w/v yeast extract (DIFCO), 8.5mM NaCl and 25mM KCl. The pH was adjusted to 7 with 5M NaOH and sterile MgCl2 solution was added to the medium just before use to give a final concentration of 25mM.

2.14.2 Generation of competent DH5α *E.coli*

To generate competent DH5α cultures, 10-12 colonies were picked from an L-agar plate and used to inoculate 250mL of SOB medium. Cultures were grown for 24-36 hours at 18-20˚C until their absorbance at 600nm reached 0.6, then were placed on ice. Bacteria were then pelleted by centrifugation at 3000rpm for 10 minutes at 4˚C. The supernatant was discarded and the pellet resuspended in 80mL filter sterilized, ice cold TB (10mM Pipes, 15mM calcium chloride ($CaCl_2$), 250mM KCl, adjusted to pH 6.7 with potassium hydroxide (KOH) and then supplemented with 55mM manganese chloride ($MnCl_2$)) and incubated on ice for 10 minutes. The culture was centrifuged again at 3000rpm for 10 minutes at 4˚C and the pellet resuspended in 20mL ice cold TB. 1.5mL of DMSO was added and the mixture incubated on ice for a further 10 minutes. 200µL aliquots of competent bacteria were snap frozen in liquid nitrogen and stored at -80˚C ready for transformation.

2.14.3 Transformation of DH5α *E.coli*

Aliquots of competent DH5α *E.coli* were thawed on ice and 100µL transferred to an autoclaved 1.5mL microcentrifuge tube containing 10ng of plasmid DNA or the product of a DNA ligation reaction. The mixture was incubated on ice for 30 minutes and then heat shocked for 1 minute at 42˚C. 1mL of L-Broth was added and the cultures incubated for an hour at 37˚C. Cells were pelleted by centrifugation at 13,000 RPM for 30 seconds, the supernatant decanted and the cells resuspended in the residual liquid. The bacteria were plated out on L-Agar/ampicillin plates and incubated overnight at 37˚C.

2.14.4 Small scale preparation of plasmid DNA

A single bacterial colony was picked using a sterile loop and used to inoculate 3mL of L-Broth containing the appropriate antibiotic (normally ampicillin). Cultures were then incubated with shaking at 37˚C overnight. The next day 1.5mL of the culture was transferred to an autoclaved 1.5mL microcentrifuge tube and the bacteria pelleted at 13,000 RPM for 1 minute. Plasmid DNA was extracted using the GE Healthcare Miniprep kit according the manufacturer's instructions and eluted in 50µL of 1x TE buffer. Digestion by restriction endonucleases (2.11) of 5µL of the sample was routinely used to check the plasmid configuration or to screen for the presence of inserted DNA.

2.14.5 Large scale preparation of plasmid DNA

A single bacterial colony was picked using a sterile loop and used to inoculate 5mL starter culture of L-Broth containing the appropriate antibiotic (normally ampicillin). The culture was then incubated with shaking at 37˚C for 8 hours, before being used to inoculate 200mL of L-Broth containing the appropriate antibiotic. This large culture was incubated with shaking at 37˚C, overnight. The next day, the bacteria were pelleted by centrifugation at 15,000 RPM for 10 minutes and plasmid DNA extracted using the maxi prep system (Jetstar) according to the manufacturer's instructions. The DNA pellet was air dried before resuspension in 200µL of TE buffer. The concentration of the plasmid DNA was determined using a Nanodrop (Thermo Scientific) according to the manufacturer's instructions. Plasmid DNA was typically diluted to 1µg/µL and stored at -20˚C until required.

2.15 Use of plasmid vectors

2.15.1 pRTS-CD2 vectors

In this study we used a number of pRTS-CD2 vectors to express genes of interest in BL cell lines. pRTS-CD2 vectors are EBV derived, episomally replicating plasmids, based on the pRTS-1 vector (Bornkamm et al., 2005). A map of the pRTS-1 expression vector is shown in Figure 2.5(A); it encodes a number of constitutively expressed genes as well as a stringent doxycycline/tetracycline control system. Expression of a gene of interest is controlled through a bidirectional doxycycline/tetracycline promoter (P_{tet}bi-1). Activity of P_{tet}bi-1 is regulated by a doxycycline (dox) controlled transcriptional activator, composed of the chicken-β-actin promoter (CAGp), flanked by the mouse Ig heavy chain intron enhancer (Eμ). The Dox controlled transcriptional activator constitutively drives expression of the activator gene, rtTA2S-M2 and silencer, tTSKRAB. Addition of dox significantly increases the binding of rtTA2S-M2 to tetO sites in the P_{tet}bi-1 promoter and thus activates transcription of the gene of interest. Conversely, the silencer, tTSKRAB binds to tetO only in the absence of dox (or tetracycline) and dissociates when an effector is added.

In pRTS-1, P_{tet}bi-1 drives bi-directional expression of enhanced green fluorescent protein (eGFP) and the firefly luciferase (luc) gene. The luc gene is flanked by unique SfiI sites, which can be used as universal cloning sites for genes of interest. Restriction enzyme digest of pRTS-1 with SfiI generates non-cohesive ends that prevent re-ligation and ensure directionality when cloning. In addition to the doxycycline response cassette, pRTS-1 also encodes the ampicillin resistance gene, β-lactamase (bla); the bacterial origin of replication (ori); an optimized hygromycin B resistance gene (hyg) driven by the SV40 early enhancer-promoter (SV40p); the EBNA1 gene driven by a cryptic promoter derived from the episomal EBV vector described in (Yates et al., 1985) and the EBV episomal origin of replication (oriP), which contains the family of repeats (FR) and dyad symmetry element (DS).

The pRTS-CD2 backbone (Figure 2.5(B)) was derived from pRTS-1 by Georg Bornkamm's lab and uses the same dox control system; however it has been subjected to a number of important modifications. The hygromycin resistance gene was replaced with the rat CD2 gene using the unique SnaBI and NotI restriction sites and the eGFP gene was replaced with a cassette containing both eGFP and the nerve growth factor receptor (NGFR) using BglII and SwaI. Expression of NGFR or rat

CD2 can be used to enrich populations of pRTS-CD2 transfected cells using magnetic activated sorting (MACS). Rat CD2 is expressed constitutively, so cells can be MACS sorted without switching on the dox promoter. Finally the luciferase gene was removed by digestion with SfiI and replaced with a gene of interest. Using this method, Georg Bornkamm developed a number of pRTS-CD2 plasmids which conditionally expressed EBV antigens including EBNA1, EBNA3B and EBNA3C.

The 3 different variants of the pRTS-CD2 backbone used in this study were pRTS-CD2, pRTS EBNA1 CD2 and pRTS EBER CD2. As stated, pRTS EBNA1 CD2 was generated by Georg Bornkamm and was kindly offered for use in this study. pRTS-CD2 was generated in our lab by Dr Gemma Kelly by manipulation of pRTS EBNA3C CD2. The EBNA3C gene was excised by SfiI digestion and the non-cohesive SfiI sticky ends were blunted and ligated together using blunt end ligation. pRTS EBER CD2 was generated by cloning the EBER genes into the pRTS-CD2 backbone as described in section 2.15.2.

Use of pRTS vectors has several advantages over traditional expression vectors for determining the effect of expression of a gene of interest on apoptosis resistance. Firstly, they are replicated and divided to daughter cells during cell division due to expression of EBNA1 and the presence of oriP. This negates the need for drug selection, which can promote the outgrowth of apoptotically resistant cells. Transfected populations can also be enriched using MACS sorting for expression of rat CD2 and NGFR or by FACS for GFP expression. Finally the presence of the GFP marker allows the effect of expression of a gene of interest to be determined in vector positive and vector negative cells from within the same population.

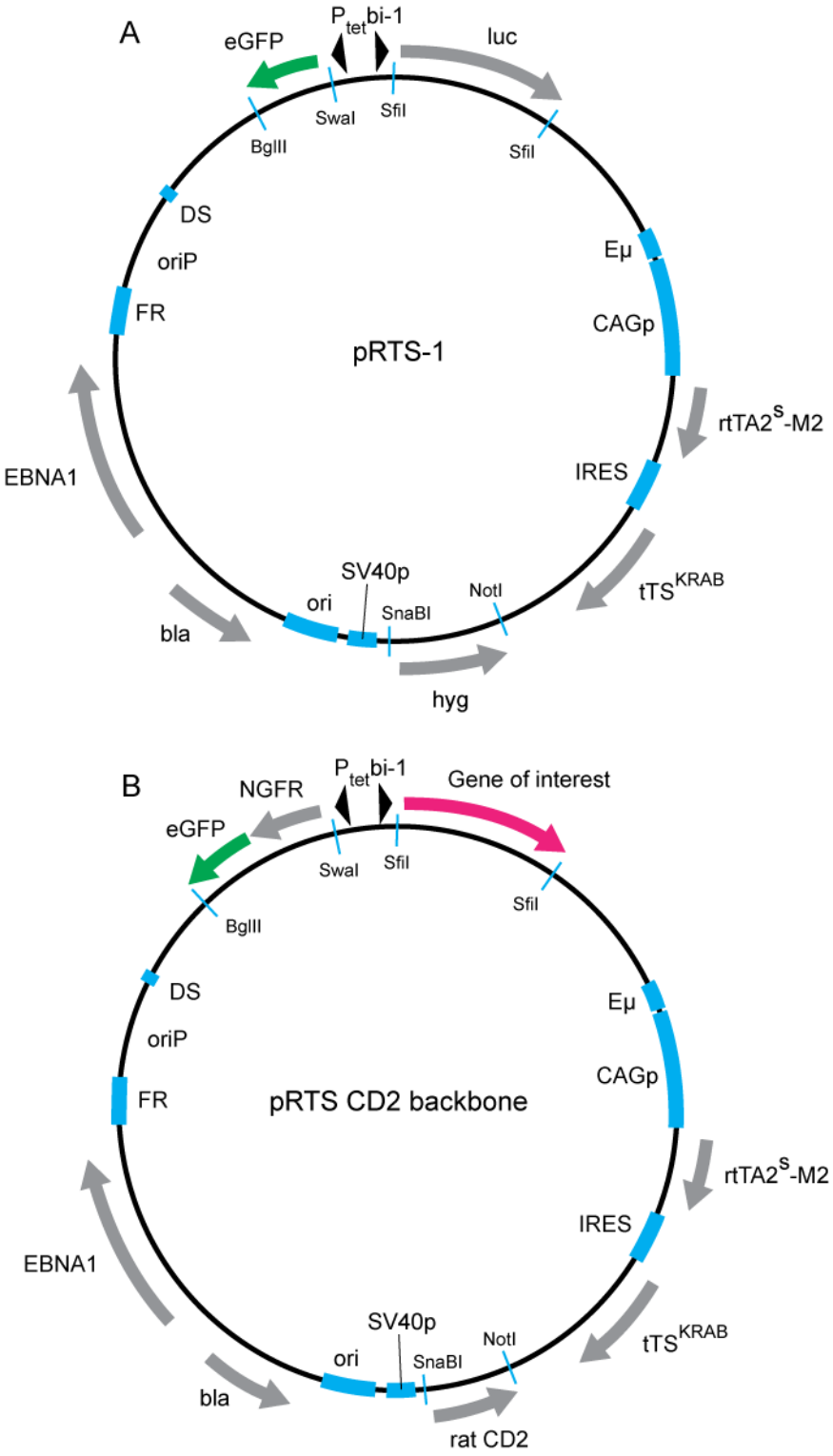


Figure 2.5. Schematic map of: (A) pRTS-1 and (B) the pRTS-CD2 backbone vector. P_{tet}bi-1 denotes the bidirectional tetracycline-regulated promoter, which activates expression of: (A) luciferase and eGFP or (B) eGFP, NGFR and a gene of interest. $rtTA^{s}$-M2 is the tetracycline controlled transactivator, tTS^{KRAB} is the tetracycline repressor and they are separated by an internal ribosomal entry site (IRES). The SV40 promoter (SV40p) drives expression of: (A) the hygromycin resistance gene (hyg) or (B) rat CD2; ori is the bacterial origin of replication; bla is β-lactamase and EBNA1 is the EBV genome maintenance protein EBNA1. OriP marks the EBV origin of replication, which contains the family of repeats (FR) and dyad symmetry element (DS).

2.15.2 Generation pRTS EBER CD2

We aimed to generate a conditional EBER1 and EBER2 expression vector based on the pRTS-CD2 system (pRTS EBER CD2). The construction of pRTS EBER CD2 is illustrated in Figure 2.6. Briefly, the EBER genes were amplified from the EBV genome by conventional PCR and cloned into the intermediate cloning vector, pUC19 SfiI. Digestion of pUC19 SfiI with SfiI generated an EBER fragment with the correct SfiI ends for directional cloning into the pRTS-CD2 backbone.

To use as a template for amplification of the EBER genes we extracted genomic DNA from the EBV transformed LCL, B95-8. We designed PCR primers to amplify a region (coordinates 6262-7343) of the B95-8 genome (de Jesus et al., 2003), which encompasses both the EBER1 and EBER2 genes and their respective promoters. Restriction sites were included in the DNA primers to allow cloning of the EBER fragment into pUC19 SfiI. The 5' primer (AAAAAAGCTTCACACTCAAGCGGGGTC) was designed with a HindIII site and a XhoI site was incorporated into the 3' primer (AAAACTCGAGTTCCTGCATGCCGTTTAATGATA).

The EBER fragment was amplified using the expand high fidelity PCR system (Roche) to reduce PCR sequence errors. Several 50µL reactions were set up containing 1x high fidelity buffer (with 1.5mM $MgCl_2$) (Roche), 200µM each dATP, dCTP, dGTP and dTTP (Roche), 1µM 5' and 3' EBER primers and 3.3 units of expand high fidelity Taq. Samples were heated to 94˚C for 5 minutes in an Eppendorf Thermocycler and incubated through 35 cycles of 94˚C for 1 minute to denature the DNA strands, 68˚C for 1 minute to allow the primers to anneal to the DNA and 72˚C for 2 minutes to allow extension of the product.

To check the size of the EBER fragment, an aliquot of the PCR product was separated by agarose gel electrophoresis (2.9) and the rest was purified by phenol chloroform extraction and sodium acetate precipitation (2.12). Purified EBER fragment and the pUC19 SfiI intermediate cloning vector were digested with HindIII and XhoI and the products purified by phenol chloroform extraction and sodium acetate precipitation. Aliquots of the digested EBER fragment and pUC19 SfiI were run on an agarose gel to check relative concentration and the EBER fragment was then ligated into the multiple cloning site (MCS) of pUC19 SfiI using a rapid ligation kit (Roche) as described in 2.13.

The pUC19 SfiI+EBERs vector was used to transform DH5α bacterial cells as described in 2.14.2 and plasmid DNA extracted from individual bacterial colonies using the miniprep system as described in

2.14.4. The presence of the EBER fragment was determined by SfiI digestion and large scale plasmid preparations were made from selected colonies (2.14.5).

The pUC19 SfiI intermediate cloning vector has been designed to contain 2 SfiI sites which flank the MCS. Digestion with SfiI generates a fragment with SfiI ends which allow directional cloning into the pRTS-CD2 backbone. To generate a pRTS-CD2 backbone with the correct SfiI sticky ends we digested the pRTS EBNA3B CD2 plasmid with SfiI and purified the product by phenol chloroform extraction and sodium acetate precipitation. The pUC19 SfiI+EBER was digested with SfiI and the products separated by agarose gel electrophoresis. The EBER fragment, flanked by the SfiI sites, was excised from the gel, purified by gel extraction (2.10) and ligated into the pRTS-CD2 backbone in the position previously occupied by the EBNA3B gene.

The product of the ligation was used to transform DH5α bacterial cells and a number of colonies were screened for the presence of the EBER fragment and general structure of the pRTS vector. Large quantities of the pRTS EBER CD2 vector were generated using the maxiprep system (2.14.5) and diluted to a concentration of 1µg/mL ready for transfection into EBV-loss cells. Figure 2.7 shows simplified maps of the pRTS-CD2, pRTS EBNA1 CD2 and pRTS EBER CD2 vectors.

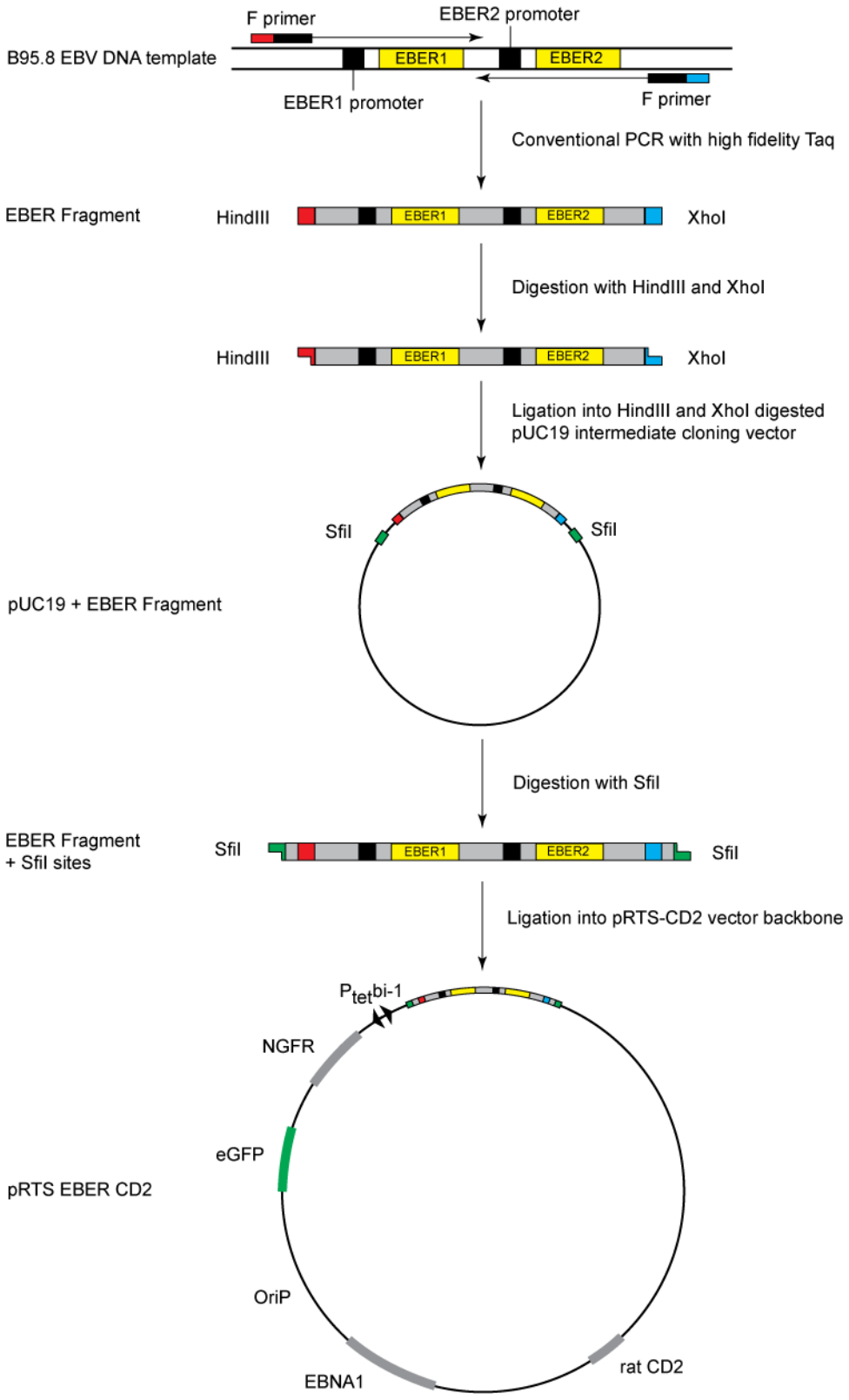


Figure 2.6. Generation of the pRTS EBER CD2 plasmid vector. An EBER fragment with HindIII and XhoI restriction sites was generated from B95-8 DNA by PCR. This EBER fragment was digested with HindIII and XhoI and ligated into the multiple cloning site of the pUC19 SfiI intermediate cloning vector. Digestion of the pUC19 EBER plasmid with SfiI produced a DNA fragment with the correct restriction sites for ligation in the correct orientation into the unique SfiI restriction site, which lies next to the bi-directional tetracycline/doxycycline promoter (Bi-tet).

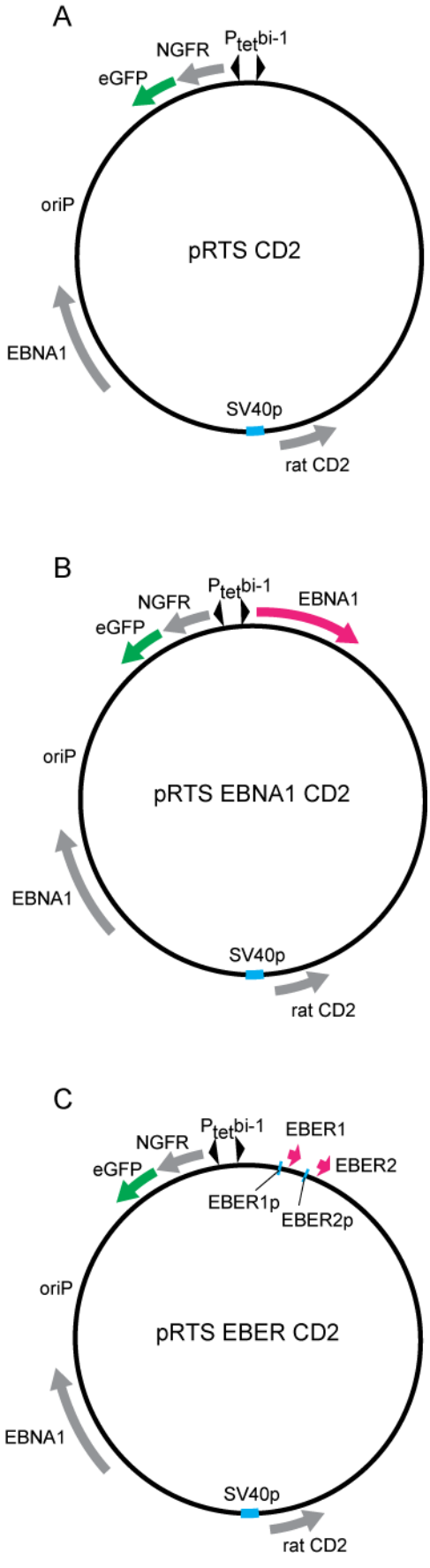


Figure 2.7. Simplified maps of: (A) pRTS-CD2. (B) pRTS EBNA1 CD2. (C) pRTS EBER CD2. P_{tet}bi-1 denotes the bidirectional tetracycline-regulated promoter, which drives expression of: (A) eGFP and NGFR (B) eGFP, NGFR and the EBV genome maintenance protein, EBNA1 (C) eGFP, NGFR and the EBERs. Expression of the rat CD2 is driven by the SV40 promoter (SV40p) and OriP marks the EBV origin of replication.

2.15.3 Transfection of pRTS-CD2 vectors into BL cells by electroporation

To establish the effect of EBNA1 and EBER expression in EBV-loss clones we used the previously described pRTS-CD2 plasmid vectors. Maximum transfection efficiency was achieved by passaging cells 24 hours before vector delivery to ensure that cells were in the optimal growth phase. Cells were washed in 1x PBS, counted and resuspended at $2x10^7$ cells/mL in Optimem medium (Gibco). 500µL of cells were transferred to a sterile 4mm electroporation cuvette (Geneflow) containing 10µg of pRTS-CD2 plasmid DNA. Electroporation was carried out using a Bio-Rad Gene Pulser II with a capacitance of 975µF and a voltage of between 270 and 290 depending on the cell line. Transfected cells were transferred immediately to 6mL of warm αTG BL cell medium and incubated at 37˚C, 5% CO_2 overnight. Dead cells and debris were frequently removed by carefully layering of cells over 3mL of lymphoprep (Axis Shield) and centrifugation at 1,600 RPM for 30 minutes without the brake. Live cells were then collected from the interphase between the BL medium and lymphoprep and washed twice in fresh BL medium. Cells transfected with pRTS vectors were frequently enriched using cell sorting. The method used to sort pRTS transfected cells depended on the cell line being sorted. MACS sorting was used to quickly generate high purity of vector-positive populations from Akata-BL cell lines, but the protocol could not be tolerated by Kem-BL cells, so vector positive cells were isolated by FACS.

2.16 Use of recombinant EBV genomes

2.16.1 Background

To restore EBV infection to EBV-loss clones, two recombinant EBV strains were used; namely the 2089 virus developed by Henri-Jacque Delecluse (Delecluse et al., 1998) and the Akata virus developed by Kenzo Takada (Shimizu et al., 1996).

The 2089 strain was made by homologous recombination in the EBV-positive, lymphoblastoid cell line, B95-8. A bacterial artificial chromosome (BAC) was developed in *E. coli*, which encoded an EBV targeting sequence along with GFP and hygromycin resistance genes. BAC DNA was transfected into B95-8 and cells which supported spontaneous homologous recombination between the wild-type virus and the BAC DNA were selected with hygromycin. Recombinant EBV genomes were extracted from B95-8 cells by circle preparation and transfected back into *E.coli*. The EBV genome was propagated

as a BAC in *E.coli* and the structure analysed by restriction enzyme digest. Finally the BAC was transfected to the EBV-negative human embryonic epithelial kidney cell line, 293. 293 cells carrying the recombinant EBV genome were selected with hygromycin and infective EBV particles produced by transfection of a plasmid encoding the EBV lytic cycle gene BZLF1.

Recombinant Akata virus was generated using a similar method in the EBV-positive sporadic BL cell line Akata-BL. BAC DNA encoding an EBV targeting sequence plus neomycin resistance and GFP genes was transfected into EBV-positive Akata-BL cells. Neomycin resistant cells were screened for the presence of recombinant EBV by southern blotting. Recombinant EBV containing cells were then induced into lytic replication by anti-immunoglobulin G (IgG) cross-linking. This generated a mixture of wild-type and recombinant virus which was used to infect EBV-negative Akata-BL cells. By single cell cloning, EBV-loss cells infected with only the recombinant Akata virus were identified and found to be highly permissive for EBV replication.

2.16.2 Production of 2089 virus from 293 cells

Infective 2089 recombinant virus particles were produced from cells from the embryonic kidney cell line, 293, which were carrying the 2089 bacterial artificial chromosome (BAC). Cells were induced into the EBV lytic cycle by transfection of a plasmid encoding the EBV immediate early lytic cycle gene, BZLF1 (Delecluse et al., 1998). This BZLF1 plasmid vector (Hammerschmidt and Sugden, 1988) was generated by ligation of the BZLF1 gene along with the promoter and enhancer for the CMV immediate-early gene promoter into a retroviral vector derived from a murine leukaemia virus.

Infectivity of 2089 virus was increased by co-transfection of a plasmid containing the EBV early lytic cycle gene, BALF4, which encodes the glycoprotein, gp110 (Neuhierl et al., 2002). The BALF4 plasmid was generated by insertion of the B95.8 BALF4 gene into the pRK5 commercial expression vector.

293 cells carrying the 2089 BAC were plated out into 6 well plates. When cells were 70% confluent the EBV lytic cycle genes were transfected into cells using lipid micelles. For each well to be transfected, a reaction mixture was prepared containing 0.5µg each of the BZLF1 and BARF4 plasmids and 250µL of Optimem (Gibco). A separate reaction mixture was also prepared containing 10µL of lipofectamine (Invitrogen) and 250µL of Optimem (Gibco) per well. The mixtures were incubated at room

temperature for 5 minutes, then mixed together and incubated for a further 20 minutes at room temperature to allow the lipofectamine to complex with the plasmid DNA. Medium was removed from 293 cells and replaced with 2mL of Optimem. After 20 minutes the Optimem was replaced with the lipofectamine/plasmid mixture and the cells incubated at 37˚C, 5% CO_2 for 3 hours. 1.5mL of RPMI+10% FCS was then added to the 293 cells and they were cultivated for a further 3 days to allow for production of infective virus particles. The supernatant was pipetted off the adherent 293 cells and any remaining cells and debris were removed by centrifugation at 1,400 RPM for 4 minutes and filtration through 0.45µm filter units. 5mL aliquots of virus supernatant were stored at -80˚C. To calculate virus titre, EBV genomes were released from viral capsids by mixing 50µL virus supernatant with 50µL virus lysis buffer (200µg/ml proteinase k in 1% v/v Tween20, 150mM Tris-HCl, 40mM $(NH_4)_2SO_4$ pH8.8), followed by 1 hour incubation at 60˚C. The number of EBV genomes per mL was then calculated using DNA-PCR for the EBV pol gene. 5µL of lysed viral supernatant were assayed by QPCR as described in 2.4.3. B2m primer and probes were not included since cellular DNA was not present. Instead values generated by comparison to the Namalwa-BL standards were multiplied by 2000 to give the number of EBV genomes per mL.

2.16.3 Production of Akata virus from Akata-BL cells

Akata virus was generated from an EBV-loss clone of Akata-BL, which had previously been reinfected with recombinant Akata virus. Approximately $50x10^6$ virus producing cells were pelleted by centrifugation at 1,400 RPM for 4 minutes and resuspended at a concentration of $4x10^6$ cells/mL in BL medium. Cells were cross-linked by 2 hours incubation with 0.5% v/v anti-human IgG Fab 2 antibody at 37˚C, 5% CO_2. The cell mixture was homogenised by gentle shaking every half hour for the duration of the incubation. Cross-linked cells were then resuspended at a concentration of $3x10^5$ cells/mL in BL medium and cultured at 37˚C, 5% CO_2, for 3 days to allow release of recombinant virus. Cells were removed from virus containing supernatant by centrifugation and filtration through 0.45µm filter units. 3mL aliquots were stored at -80˚C and virus titre determined using quantitative DNA-PCR for the EBV pol gene as described above.

2.16.4 Infection of BL cells with recombinant EBV

$1x10^6$ EBV-negative BL cells were incubated overnight with $50x10^6$ EBV particles at 37˚C, 5% CO_2. Cells were pelleted, resuspended in fresh BL medium and cultured for a further 24-48 hours to allow expression of recombinant viral proteins. Cells were then sorted by FACS for GFP expression into wells of 96 well plates or drug selected to generate bulk cultures using 100µg/mL hygromycin (Gibco) for 2089 virus infected cells or 100µg/mL geneticin (Invitrogen) for Akata virus infected cells.

2.17 Cell sorting

Cell sorting was used to enrich populations of cells infected with recombinant EBV or transfected with pRTS-CD2 plasmid vectors. EBV-infected cells were sorted on GFP expression using fluorescence activated cell sorting (FACS). EBV-loss clones transfected with pRTS-CD2 vectors were sorted on expression of rat-CD2 using magnetic activated cell sorting (MACS) or on expression of GFP using FACS.

2.17.1 Fluorescence activated cell sorting

Fluorescence activated cell sorting (FACS) was used to enrich populations of cells infected with recombinant EBV or transfected with pRTS vectors based on expression of GFP. FACS was carried out using the cell sorting service at the Institute for Biomedical Research (IBR). Cells were sorted on GFP expression using a MoFlo cell sorter (Beckman Coulter).

To isolate EBV-infected cells, EBV-loss clones were infected with 2089 or Akata virus as described in 2.16.4. Infected cells were then incubated at 37˚C in normal BL medium for 48 hours to allow expression of virally encoded GFP. FACS was then used to sort individual GFP-positive cells into wells of 96 well round bottom plates containing feeder layers of $2x10^3$ human fibroblasts.

To enrich Kem-BL cells transfected with pRTS vectors, transfected cells were cultivated for 2 weeks to generate large numbers of cells. 48 hours prior to sorting $3x10^7$ cells were induced with 50ng/mL doxycycline to activate the bi-directional promoter and induce expression of GFP and the gene of interest. The cells were pelleted by centrifugation at 1,400 RPM for 4 minutes and resuspended to $3x10^7$ cells/mL in BL medium. Purified populations of GFP-positive and GFP-negative cells were

isolated by FACS and incubated for at least 48 hours, at 37˚C, 5% CO_2, to allow recovery from FACS. Aliquots of pRTS vector positive and pRTS vector negative cells were harvested for protein and RNA determination and apoptosis was induced with ionomycin and anti-IgM cross-linking.

2.17.2 Magnetic activated cell sorting

Magnetic activated cell sorting (MACS) was used to enrich populations of Akata-BL cells transfected with pRTS plasmids based on expression of rat-CD2. Plasmid vectors were delivered by electroporation and live cells isolated using lymphoprep (2.15.3). Cells were washed twice in BL medium to remove residual lymphoprep and pelleted by centrifugation at 1,400 RPM for 4 minutes. Transfected cells were resuspended in 100µL 1x PBS containing 10µg/mL OX34 anti-rat-CD2 primary antibody and placed on ice. After 30 minutes cells were washed twice in 5mL of ice cold MACS buffer (5mM EDTA, 0.5% BSA in 1x PBS) and resuspended in 80µL of MACS buffer and 20µL of Rat anti-mouse IgG2a+b microbeads (Miltenyi Biotec). Cells were transferred to an autoclaved 1.5mL microcentrifuge tube and incubated with gentle rotation at 4˚C for 15 minutes. 900µL of MACS buffer was added and cells were pelleted at 3,000 RPM for 3 minutes before resuspension in 1mL of ice cold MACS buffer. Microbead labelled cells were applied to a LS MACS separation column (Miltenyi Biotec), which had been prerinsed with 5mL of ice cold MACS buffer, connected to a QuadroMACS magnetic separator. Rat CD2-negative cells were washed through the column by the addition of 2x 10mL of ice cold MACS buffer. The LS column was then removed from the QuadroMACS magnetic separator and CD2-positive cells eluted in 5mL of BL medium. Any residual liquid in the column was gently forced through the column with a plunger. Cells were pelleted by centrifugation, resuspended in warm BL medium and incubated at 37˚C, 5% CO_2. CD2-positive cells were expanded until enough cells were present to investigate expression of EBNA1 and/or EBERs and to induce into apoptosis. Aliquots of cells were induced with a range of concentrations of doxycycline. Cells were then harvested for protein and RNA determination and apoptosis was induced with ionomycin and roscovitine.

3. Results Part I

Generation of EBV-loss clones from EBV-positive eBL cell lines

3.1 Introduction

Despite the long established link between EBV and endemic Burkitt Lymphoma (eBL) (Epstein et al., 1964a), the role of EBV in tumour initiation and progression remains elusive. An opportunity to study the effect of EBV in a BL cell environment came with the first reports of spontaneous loss of EBV from the EBV-positive, sporadic BL cell line, Akata-BL (Shimizu et al., 1994). Loss of EBV indicated that, in Akata-BL, EBV is not absolutely required for BL cell proliferation. Furthermore, comparison of pure EBV-positive and EBV-loss (EBV-negative) populations generated by single cell cloning demonstrated that despite the ability to proliferate in normal culture conditions, EBV-loss clones had reduced survival ability in low serum and soft agar and a reduced resistance to apoptosis (Komano et al., 1998). The aim of the first part of the study was to investigate EBV-loss in a panel of endemic BL cell lines and determine its effect on cell phenotype.

3.2 Single cell cloning of Burkitt Lymphoma cell lines

In the first series of experiments, we aimed to confirm the earlier observation that Akata-BL could spontaneously lose the EBV genome and then attempted to isolate EBV-loss clones from eBL cell lines. We selected a panel of 12 EBV-positive, Latency I, BL cell lines for single cell cloning. These included a late passage culture of the sporadic, BL cell line Akata-BL and 11 eBL cell lines from early, intermediate and late passage.

BL cell lines were cultured in medium supplemented with acyclo-guanosine for 2 weeks to inhibit the production of infectious EBV virions. Acyclo-guanosine, also known as acyclovir (ACV), is an analogue of the nucleotide, guanine, which is converted to acyclo-guanosine triphosphate (acyclo-GTP) by a combination of cellular and virally encoded kinases. Acyclo-GTP is a potent inhibitor of DNA polymerase, which affects the viral enzyme around 100 times more effectively than the cellular

enzyme. Incorporation of Acyclo-GTP into viral DNA by viral DNA polymerase results in chain termination and thus inhibits lytic replication.

BL cells were then single cell cloned on to fibroblast feeder layers (as described in section 2.1.3). DNA was extracted from newly generated clones and screened for the presence of EBV using a quantitative PCR (QPCR) assay for the EBV pol gene (section 2.4.3). Representative QPCR data, showing EBV viral load in a panel of clones derived from the single cell cloning of 2 early passage eBL cell lines (Dante-BL and Mutu-BL), is shown in Figure 3.1. All 95 clones of Dante-BL screened were EBV-positive; on average each clone contained around 25 viral genomes per cell. The number of EBV genomes per cell did not vary greatly between different Dante-BL clones, perhaps suggesting stable segregation of EBV genomes to daughter cells during cell division. The average viral load in clones of Mutu-BL was much higher (around 65 EBV genomes per cell). However this was accompanied by a much greater variation in viral load across different clones. Most clones contained between 5 and 150 EBV genomes per cell. A single clone had a viral load of 340 EBV genomes per cell; this is likely to be a result of EBV lytic replication, despite the treatment with ACV. Importantly, of the 195 clones of Mutu-BL examined, 3 were found to be completely negative for EBV and these are marked by a * on Figure 3.1(B).

The results from single cell cloning of the remaining BL lines are shown in Table 7. In total we examined 3 early passage cell lines (passage 1-20), 7 intermediate passage cell lines (passage 21-40) and 4 late passage cell lines (passage 41+). From the 3 early passage cell lines (Sav, Dante and Mutu), we were only able to isolate EBV-loss clones from Mutu-BL. From the 7 intermediate passage eBL cell lines investigated (Chep-BL, Sal-BL, Oku-BL, Kem-BL, Eli-BL, Ava-BL and Awia-BL), only Ava-BL and Awia-BL generated EBV-loss clones. Ava-BL yielded only a single EBV-loss clone, which was not viable without the support of a fibroblast feeder layer, but 2 EBV-loss clones were isolated from Awia-BL which were viable under normal culture conditions. Finally we investigated the effect of serial passage *in vitro* on the rate of EBV-loss by single cell cloning 3 late passage eBL cell lines (Rael-BL, Eli-BL and Kem-BL). No EBV-loss clones were isolated from 91 clones of Rael-BL screened; however both Eli-BL and Kem-BL yielded EBV-loss clones, despite the fact that they were unable to do so in an earlier passage. In addition to these eBL cell lines, we also confirmed the earlier observation of EBV-loss in the sporadic BL cell line, Akata-BL.

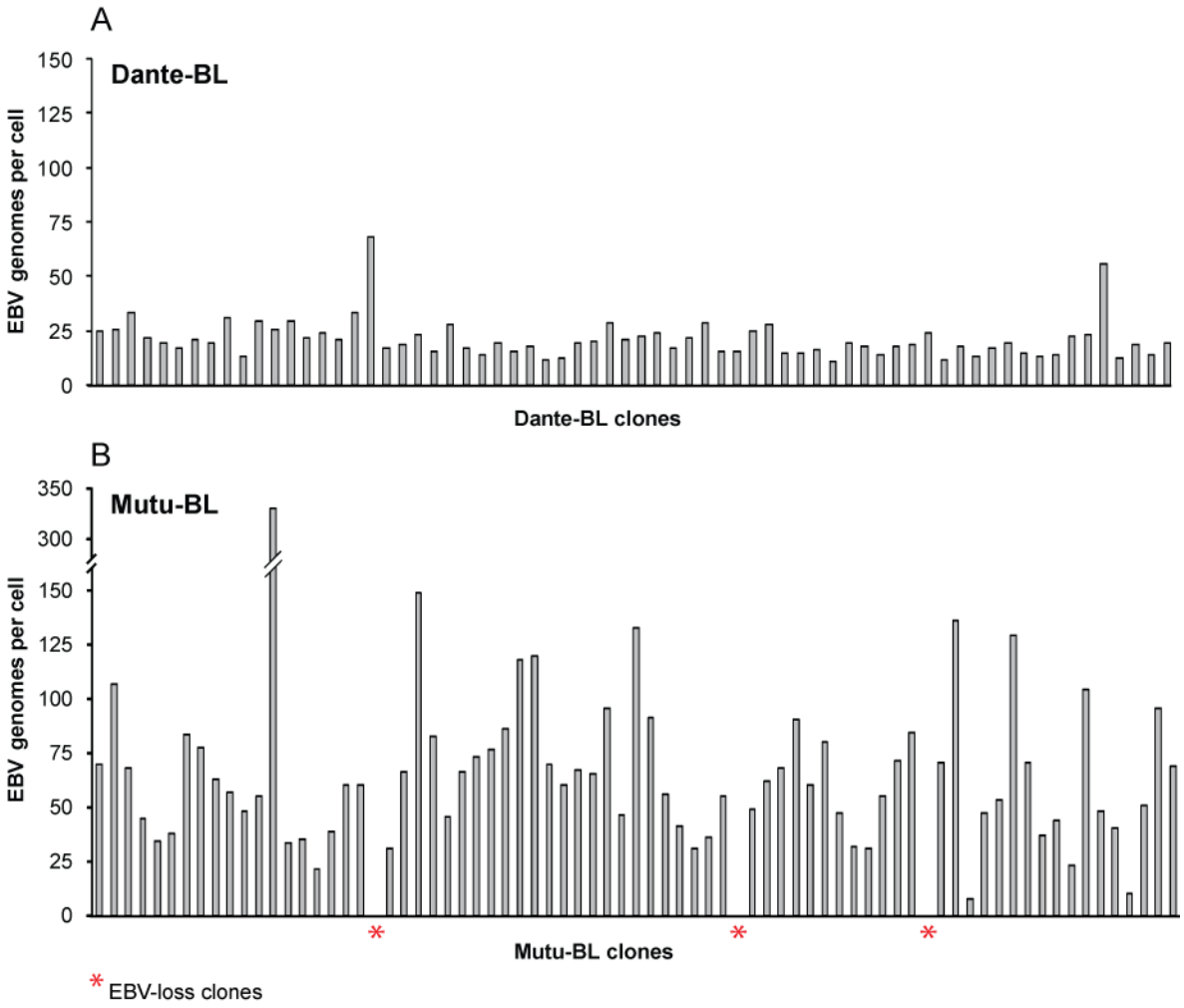


Figure 3.1. Representative viral load data from a panel of clones generated by the single cell cloning of early passage eBL cell lines (A) Dante-BL and (B) Mutu-BL. EBV genome load was determined by quantitative DNA-PCR for the EBV pol gene in single cell clones and the EBV-loss clones generated from Mutu-BL are marked with a *.

Cell line cloned	Passage number	Mean viral load	No. of clones screened	EBV-positive clones	EBV-loss clones	Viral load range
	Early passage					
Sav-BL	p7	165	175	175	0	30 - >500
Dante-BL	p17	21	95	95	0	11 - 68
Mutu-BL	p4	64	195	192	3 (1.5%)	0 - 150
	Intermediate passage					
Chep-BL	p24	12	149	149	0	2-80
Sal-BL	p35	17	72	72	0	6-30
Oku-BL	p36	10	135	135	0	5-37
Kem-BL	p27	82	102	102	0	5 - >300
Eli-BL	p36	26	21	21	0	6 - 68
Ava-BL*	p27	65	158	157	1 (0.6 %)	0 - 68
Awia-BL	p40	10	192	190	2 (1%)	0 - 35
	Late passage					
Rael-BL	>200	124	91	91	0	7 - >300
Eli-BL	>200	13	120	82	38 (32%)	0 - 48
Kem-BL	p120	26	44	43	1 (2%)	0 - 75
Akata-BL**	>200	16	45	40	5 (11%)	0 - 51

Table 7. Generation of EBV-loss clones from a panel of early, intermediate and late passage BL cell lines by single cell cloning. With the exception of sporadic BL cell line Akata-BL, all cell lines were derived from endemic BL tumours. * The single EBV-loss clone generated from Ava-BL did not survive. ** Published data on early passage Akata-BL indicates that it does not yield EBV-loss clones (Shimizu et al., 1994).

3.3 Characterisation of EBV viral latency in newly generated BL clones

Most EBV-positive BL cell lines cultured *in vitro* retain the restricted Latency I viral gene expression pattern seen in BL tumours, the genome maintenance protein EBNA1 being the only viral protein expressed from a Q-U-K spliced transcript driven from the Q promoter (Qp). Latency I cells also express the non-coding EBERs, the *Bam*HI-A rightward transcripts (BARTs) and a number of EBV encoded miRs from their own promoters.

In culture, some BL cell lines drift from Latency I to the full Latency III growth transforming programme seen in lymphoblastoid cell lines (LCLs). The switch from Latency I to Latency III is accompanied by silencing of Qp and activation of the C and W promoters (Cp and Wp). In Latency III cells, EBNA1 is expressed as a Y_3-U-K transcript, initiated from Cp or Wp. Cp and Wp initiated transcripts also encode the EBNA-LP, EBNA2 and the EBNA3A, 3B and 3C proteins, which in turn leads to expression of the latent membrane proteins (LMP1 and LMP2) from their own promoters. Expression of these additional Latency III transcripts in BL cells is accompanied by a distinct change in morphological appearance. The small, single, spherical BL cells become enlarged and irregular and form clumps, which are visible to the naked eye. Cells in EBV-positive BL cultures may also enter the EBV lytic cycle through activation of the immediate early lytic cycle genes. During the lytic cycle, EBNA1 is produced from the lytic F promoter (Fp) found upstream of Qp and expression of a number of lytic cycle transcripts results in the production of infective viral particles. In the following sections we analysed EBV viral latency in our newly generated BL clones.

3.3.1 EBV viral latency in clones of Akata-BL

Single cell cloning of Akata-BL generated 40 EBV-positive clones and 5 EBV-loss clones as determined by quantitative DNA-PCR for the EBV pol gene. We selected 3 EBV-positive clones with a range of viral loads (ap1, ap2 and ap3) and 3 EBV-loss clones (an1, an2 and an3) for further investigation.

In the first instance, genomic DNA was prepared from selected Akata-BL clones and EBV viral load re-analysed by DNA-QPCR (Figure 3.2(A)). As expected all 3 EBV-positive clones had detectable EBV loads (25-125 EBV genomes per cell), while EBV was undetected in the EBV-loss clones. We noticed

that the EBV viral load in EBV-positive clones had increased from the values seen during the initial screening of Akata-BL clones during single cell cloning, which may represent low level activity of the EBV lytic cycle.

To confirm that every cell in the EBV-positive clones carried EBV and that EBV-loss clones were truly negative, we screened for EBER expression using a flow cytometric assay. EBER expression represents the gold standard for EBV positivity in single cells and so this assay allowed us to determine the percentage of EBV-positive cells in each Akata-BL clone. Figure 3.2(B) shows flow cytometric data from Akata-BL clones after staining with a fluorescent, peptide nucleic acid (PNA) EBER probe. As controls we used the EBV-positive, BL cell line, Raji-BL (shown to contain around 50 copies of EBV per cell (Anvret et al., 1984; Tierney et al., 2007) and the EBV-negative, B cell lymphoma cell line, Bjab. The threshold of detection for EBER positive cells in the EBV-negative Bjab cells was set at 2%. Using this baseline, we found EBER staining in 85% of Raji-BL cells. The remaining 15% of unstained Raji-BL cells may represent dead or dying cells, in which EBER transcripts would have been degraded. Staining in the Akata-BL parental cell line was found to be slightly lower than in Raji-BL cells at 77%, possibly reflecting the presence of a population of EBV-loss cells. EBER staining in EBV-positive clones was comparable with the staining found in Raji-BL, indicating that a very high percentage of cells were EBV-positive. In EBV-loss clones staining was found to be equivalent to the level observed in Bjab cells, confirming the results of the DNA-PCR analysis.

Having confirmed the EBV status of Akata-BL clones, we wished to ask if EBV-positive cells expressed the same restricted pattern of latent gene expression (Latency I) as seen in the parental Akata-BL cell line. We therefore used western blotting to screen for the expression of EBNA1 and the major growth transforming proteins EBNA2 and LMP1. Figure 3.2(C) shows the expression of EBNA1, EBNA2 and LMP1 in Akata-BL and the selected EBV-positive and EBV-loss clones. The EBV transformed LCL, X50-7, was used as a positive control for expression of all three latent viral proteins and Bjab was used as a negative control. As expected, no EBV-encoded proteins were detected in EBV-loss clones. Akata-BL and the EBV-positive clones expressed EBNA1 at a slightly lower level than X50-7, but were completely negative for EBNA2 and LMP1. Thus it is likely that EBV-positive clones retained the same Latency I viral gene expression as the parental Akata-BL culture.

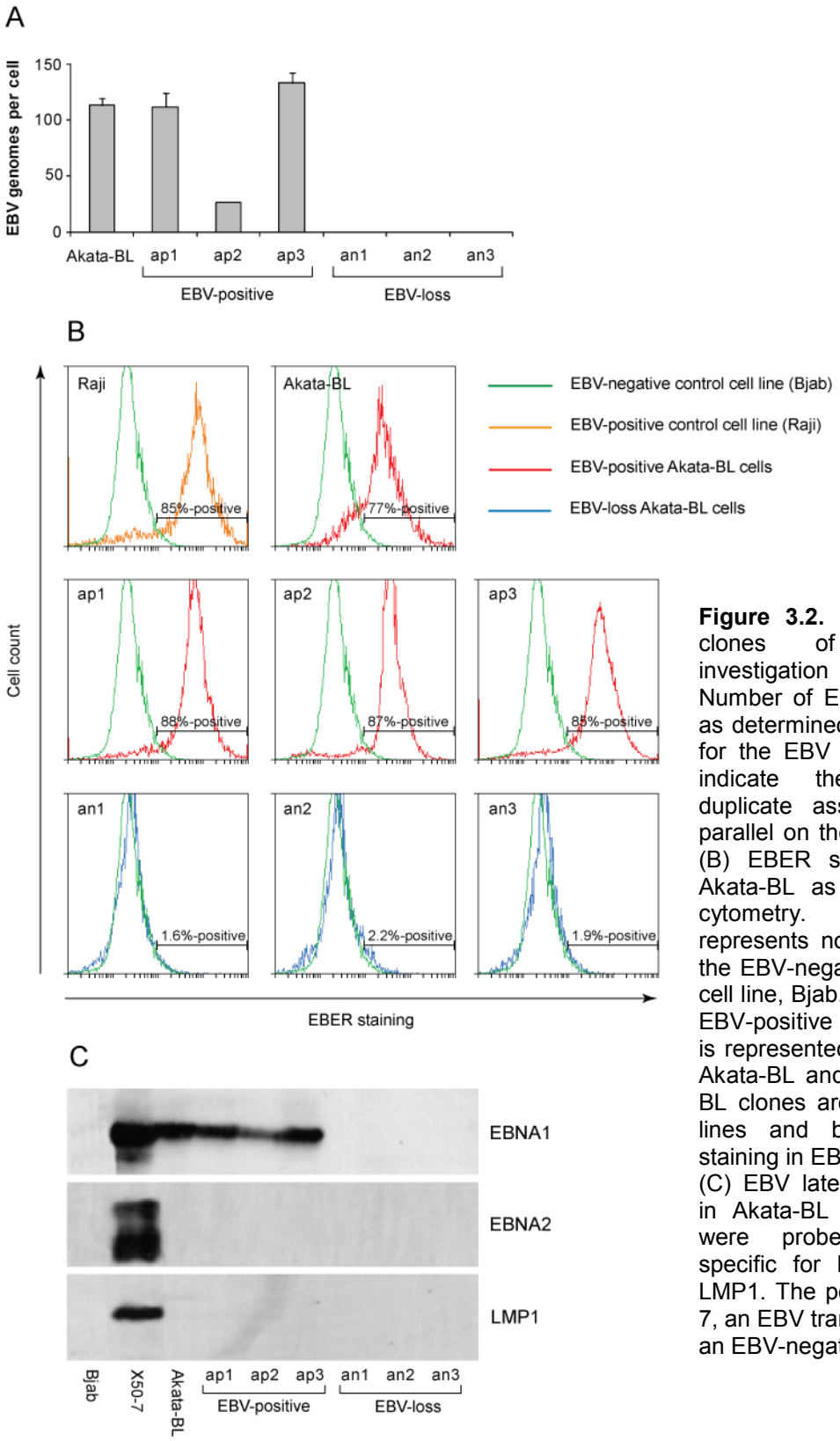


Figure 3.2. Detection of EBV in clones of Akata-BL and investigation of viral latency. (A) Number of EBV genomes per cell as determined by quantitative PCR for the EBV pol gene. Error bars indicate the range between duplicate assays carried out in parallel on the same DNA sample. (B) EBER staining in clones of Akata-BL as determined by flow cytometry. The green line represents non-specific staining in the EBV-negative B cell lymphoma cell line, Bjab. EBER staining in the EBV-positive control cell line, Raji is represented with an orange line. Akata-BL and EBV-positive Akata-BL clones are represented by red lines and blue lines represent staining in EBV-loss clones.
(C) EBV latent protein expression in Akata-BL clones. Immunoblots were probed with antibodies specific for EBNA1, EBNA2 and LMP1. The positive control is X50-7, an EBV transformed LCL. Bjab is an EBV-negative B cell lymphoma.

To confirm this observation, we used QRT-PCR to investigate viral promoter usage and specific latent viral transcripts. Analysis of Akata-BL cells by QRT-PCR is shown in Figure 3.3. Initially we determined the activity of the Latency I Q promoter by investigating Q-U-K spliced EBNA1 transcripts. We found that all EBV-positive clones expressed Q-U-K transcripts at a level comparable with the control cell line Rael-BL. By EBER flow cytometry, EBV-positive Akata-BL clones were already known to express EBERs; we therefore used 2 separate QRT-PCR assays to individually quantify levels of EBER1 and EBER2. EBV-positive clones were found to express high levels of both EBER1 and EBER2. Interestingly, when compared to the EBV transformed LCL, X50-7, they expressed around 8-fold higher levels of EBER1 compared to EBER2; however the significance of this result is unclear.

Akata-BL clones were negative or had barely detectable expression from the latency III associated promoters, Cp or Wp, and we also used QRT-PCR to confirm a lack of EBNA2 and LMP1 transcripts (data not shown). Qp activity in the absence of Cp or Wp transcripts confirms that the vast majority of EBV-positive Akata-BL clones have retained the Latency I viral gene expression pattern seen in the parental Akata-BL cell line.

Finally, we sought to determine whether any cells within EBV-positive clones were carrying EBV in a lytic rather than a latent state. We measured the activity of the lytic Fp promoter and expression of the immediate early lytic cycle gene BRLF1 using QRT-PCR. Expression of F-U transcripts from Fp is particularly important to quantify as the QRT-PCR assay for Q-U-K detects both F-U and Q-U-K spliced transcripts. Low level expression of F-U and BRLF1 was detected in Akata-BL and all EBV-positive Akata-BL clones. However this represented only 20% of the level observed in the lytic LCL control cell lines (Sal-LCL and IMI05), which themselves have only 2% of cells in lytic cycle. This implies that only a very small percentage of cells are in active lytic cycle.

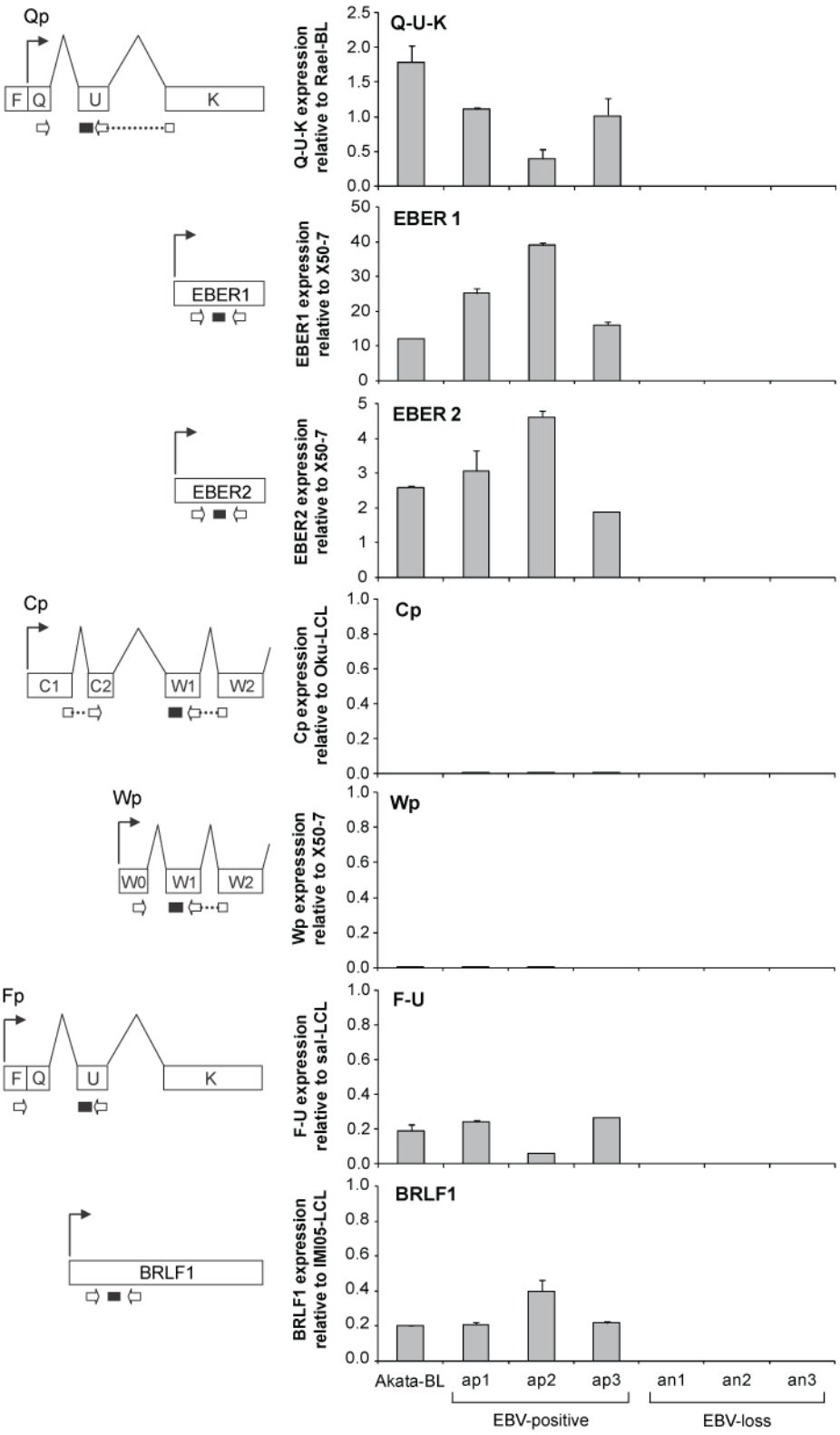


Figure 3.3. EBV gene expression in clones of Akata-BL as determined by quantitative RT-PCR. Transcription levels were detected using the primer and probe combinations indicated. The hollow arrows represent primers and the black boxes are the TaqMan probes. Transcription levels were measured relative to an appropriate control (assigned a value of 1) and error bars indicate the range between duplicate assays carried out in parallel on the same cDNA sample.

3.3.2 EBV viral latency in clones of Mutu-BL

As for clones of Akata-BL, clones of Mutu-BL were investigated for EBV viral load by quantitative DNA-PCR for the EBV pol gene and for expression of EBV latent antigens by western blotting and QRT-PCR. The analysis of EBV latency is particularly important in the context of Mutu-BL clones, since they have been previously shown to drift from Latency I to Latency III during cell culture (Gregory et al., 1991). This shift from Latency I to Latency III was accompanied by an increased resistance to apoptosis, hence we also aimed to isolate Latency III clones to use as controls for investigation into the effect of EBV on the phenotype of Mutu-BL cells.

Single cell cloning of Mutu-BL generated 195 clones. As shown by the representative data in Figure 3.1, there was a large variation in the EBV viral load between Mutu-BL clones; three clones were isolated which had completely lost EBV. Most EBV-positive Mutu-BL clones retained the single-cell, spherical appearance seen in the parental BL cell line. However, the cells of a significant number of clones became enlarged and irregular and cells formed clumps similar to those seen in lymphoblastoid cell lines (LCLs). We selected the 3 EBV-loss clones, along with 3 single-cell clones and 3 LCL-like clones for further analysis.

Figure 3.4(A) shows the EBV genome load as determined by DNA-QPCR in the 9 selected Mutu-BL. EBV-positive clones had on average between 10 and 80 EBV genomes per cell and there appeared to be no difference in EBV genome load between single-cell and LCL-like clones. EBV-loss clones were also confirmed to be completely EBV-negative.

Western blotting for EBNA1, EBNA2 and LMP1 in Mutu-BL clones is shown in Figure 3.4(B). Single-cell clones expressed EBNA1 at a level slightly lower than the EBV transformed LCL, X50-7, but no EBNA2 or LMP1 protein could be detected. However, LCL-like clones expressed similar levels of EBNA1, EBNA2 and LMP1 to X50-7. This indicates that single-cell clones retained Latency I viral gene expression, while LCL-like clones had drifted to Latency III.

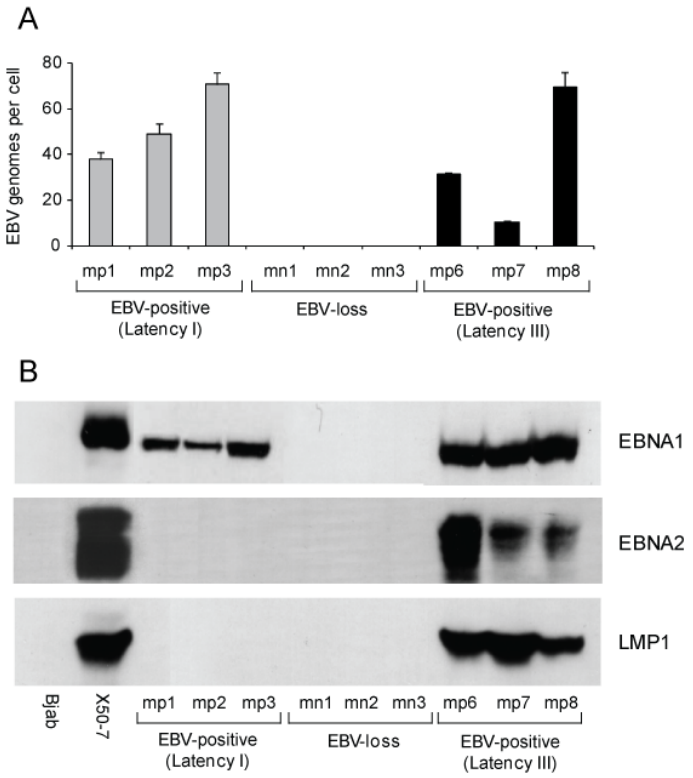


Figure 3.4. Detection of EBV in clones of Mutu-BL and investigation of viral latency. (A) Number of EBV genomes per cell as determined by quantitative PCR for the EBV pol gene. Error bars indicate the range between duplicate assays carried out in parallel on the same DNA sample. (B) EBV latent protein expression in Mutu-BL clones. Immunoblots probed with antibodies specific for EBNA1, EBNA2 and LMP1. The positive control is X50-7, an were EBV transformed LCL. Bjab is the EBV negative control cell line.

To confirm this, we used QRT-PCR to investigate viral promoter usage and expression of latent and lytic viral transcripts. Analysis of Mutu-BL cells by RT-PCR is shown in Figure 3.5. Qp activity could be detected in all EBV-positive clones, although levels of Q-U-K transcripts were lower than the control cell line, Rael-BL. All EBV-positive clones also expressed EBER1 and EBER2 at a level several fold higher than the X50-7 LCL. As was observed in clones of Akata-BL, Mutu-BL clones also appeared to express higher EBER1 transcripts compared to EBER2. Restricted Latency I viral gene expression was confirmed in single-cell clones by a lack of expression from Cp and Wp. They were also negative for EBNA2 and LMP1 transcripts (data not shown). In contrast, we found high levels of Cp and Wp transcripts in LCL-like clones along with expression of the EBNA2 and LMP1 genes.

When EBV lytic transcripts were analysed in Mutu-BL clones, very few Latency I cells appeared to be in active lytic cycle. There was negligible activity from Fp and although BRLF1 transcripts could be detected, they were only a fraction of the level detected in the lytic LCL control cell line, IMI05. The drift from Latency I to Latency III in LCL-like clones, however, resulted in the activation of the lytic EBNA1 F promoter (Fp) and high level expression of the early lytic cycle transcript, BRLF1. It is likely that these Fp initiated F-U transcripts account for the apparent activity of Qp in these clones.

EBV-positive Latency I clones (mp1, mp2 and mp3) contained high numbers of EBV genomes after single cell cloning and remained EBV-positive for several months in culture. After this time however, we noticed that cells began to lose EBV and eventually cultures became largely EBV-negative. All experiments using these clones were carried out while the vast majority of cells in these cultures were EBV-positive. However, to avoid any use of potentially mixed EBV-positive and EBV-loss populations these clones were replaced for some experiments with 2 independently established, Latency I Mutu-BL clones (mp4 and mp5).

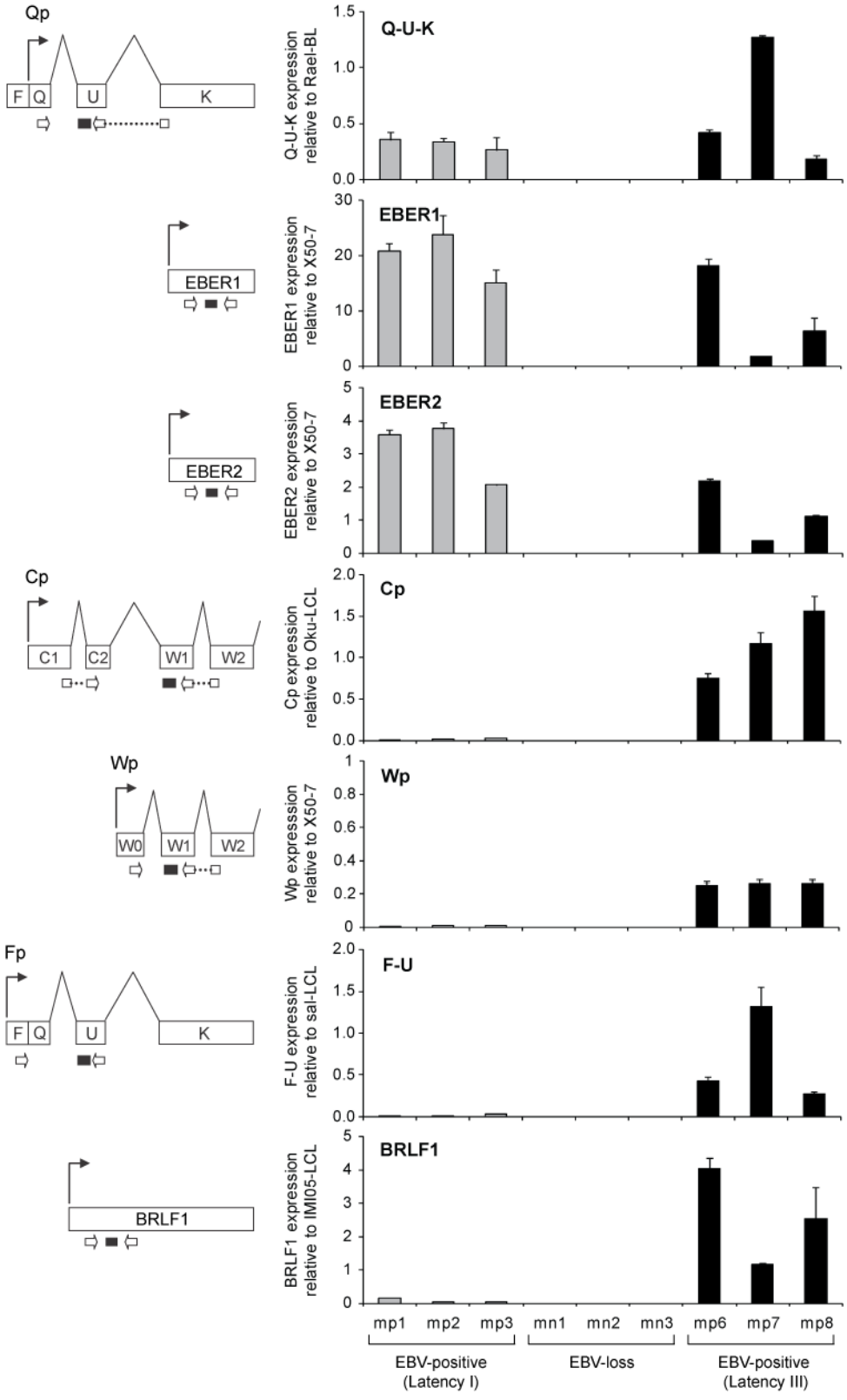


Figure 3.5. EBV gene expression in clones of Mutu-BL as determined by quantitative RT-PCR. Transcription levels were detected using the primer and probe combinations indicated. The hollow arrows represent primers and the black boxes are the TaqMan probes. Transcription levels were measured relative to an appropriate control (assigned a value of 1) and error bars indicate the range between duplicate assays carried out in parallel on the same cDNA sample.

EBV-positive Mutu-BL clones, mp4 and mp5, were investigated using the same assays as were used to investigate the original EBV-positive clones (mp1, mp2 and mp3). In addition, to ensure that all the cells within the culture carried viral genomes, we determined the percentage of EBER positive cells using flow cytometry for the EBERs. As shown in Figure 3.6 and Figure 3.7, these new clones were indistinguishable from clones mp1, mp2 and mp3 in terms of viral gene expression. Using quantitative DNA-PCR for the EBV pol gene (Figure 3.6(A)), we found that clones mp4 and mp5 had an average genome load of 150 and 75 EBV genomes per cell respectively. EBER staining (Figure 3.6(B)) revealed that the percentage of EBV-positive cells in these clones was comparable to that of Raji-BL, indicating that they stably maintain the genome. By western blot (Figure 3.6(C)), mp4 and mp5 expressed only the genome maintenance protein EBNA1, in the absence of EBNA2 or LMP1, indicating Latency I viral gene expression. This was confirmed using QRT-PCR to investigate viral promoter usage and specific latent and lytic viral transcripts. As shown in Figure 3.7, mp4 and mp5 expressed Qp derived Q-U-K transcripts at around 30% of the level observed in Rael-BL and expressed EBER1 and EBER2 at a level several times higher than X50-7 LCL. By contrast, there was little or no expression from the Latency III promoters, Cp and Wp, and no expression of EBNA2 or LMP1 (data not shown). EBV lytic F-U and BZLF-1 transcripts were seen at very low levels; around 10% of the level observed in the 2% lytic control cell lines. Thus, these alternative clones provide a highly suitable replacement for the EBV-positive clones mp1, mp2 and mp3 for investigation into the effect of EBV in Mutu-BL.

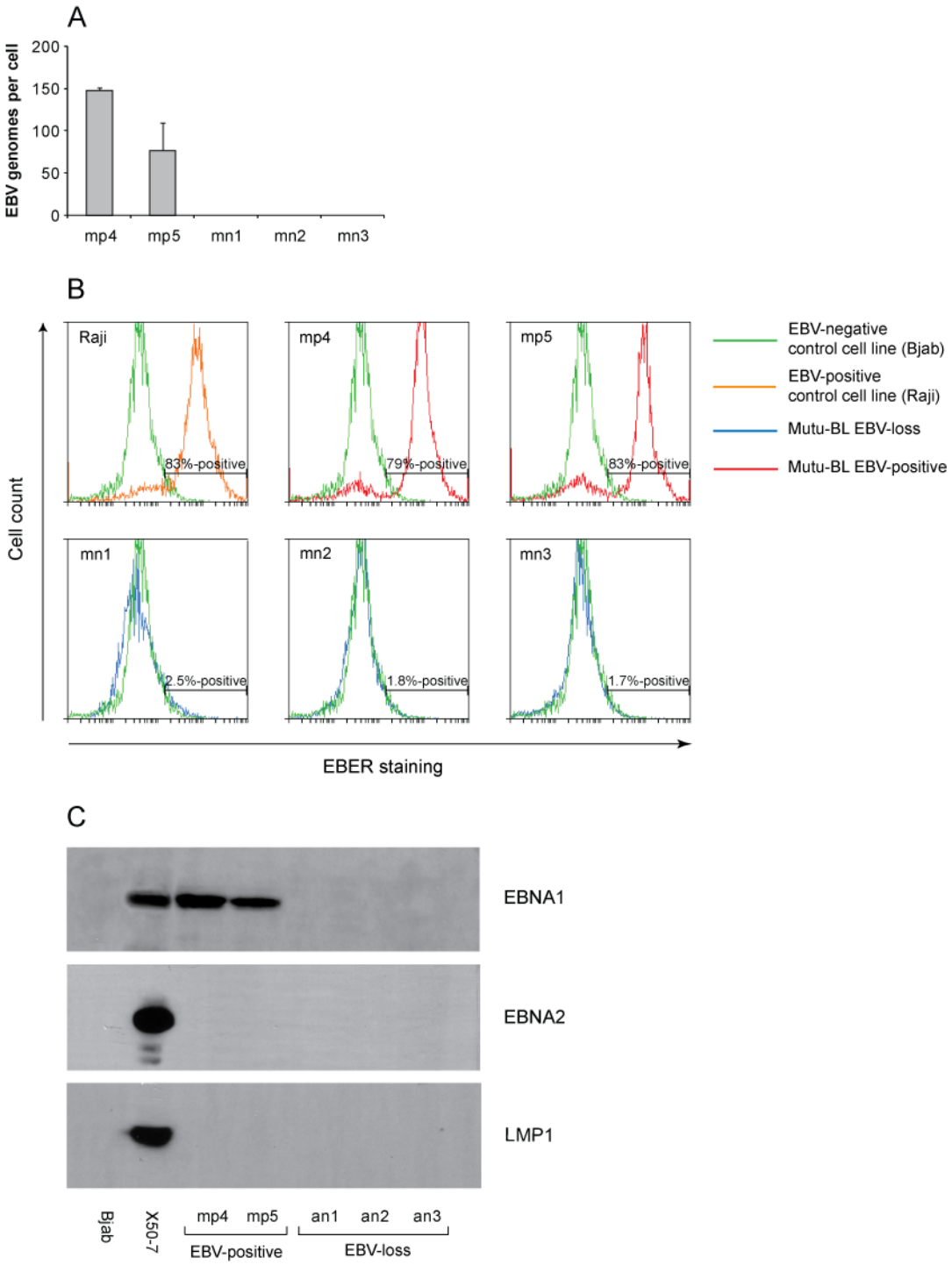


Figure 3.6. Detection of EBV in clones of Mutu-BL and investigation of viral latency. (A) Number of EBV genomes per cell as determined by quantitative PCR for the EBV pol gene. Error bars indicate the range between duplicate assays carried out in parallel on the same DNA sample. (B) EBER staining in clones of Mutu-BL as determined by flow cytometry. The green line represents non-specific staining in the EBV-negative B cell lymphoma cell line, Bjab. EBER staining in the EBV-positive control cell line, Raji, is represented with a orange line. EBV-positive Mutu-BL clones are represented by red lines and blue lines represent staining in EBV-loss clones. (C) EBV latent protein expression in Mutu-BL clones. Immunoblots were probed with antibodies specific for EBNA1, EBNA2 and LMP1. The positive control is X50-7, an EBV transformed LCL. Bjab is an EBV-negative B cell lymphoma.

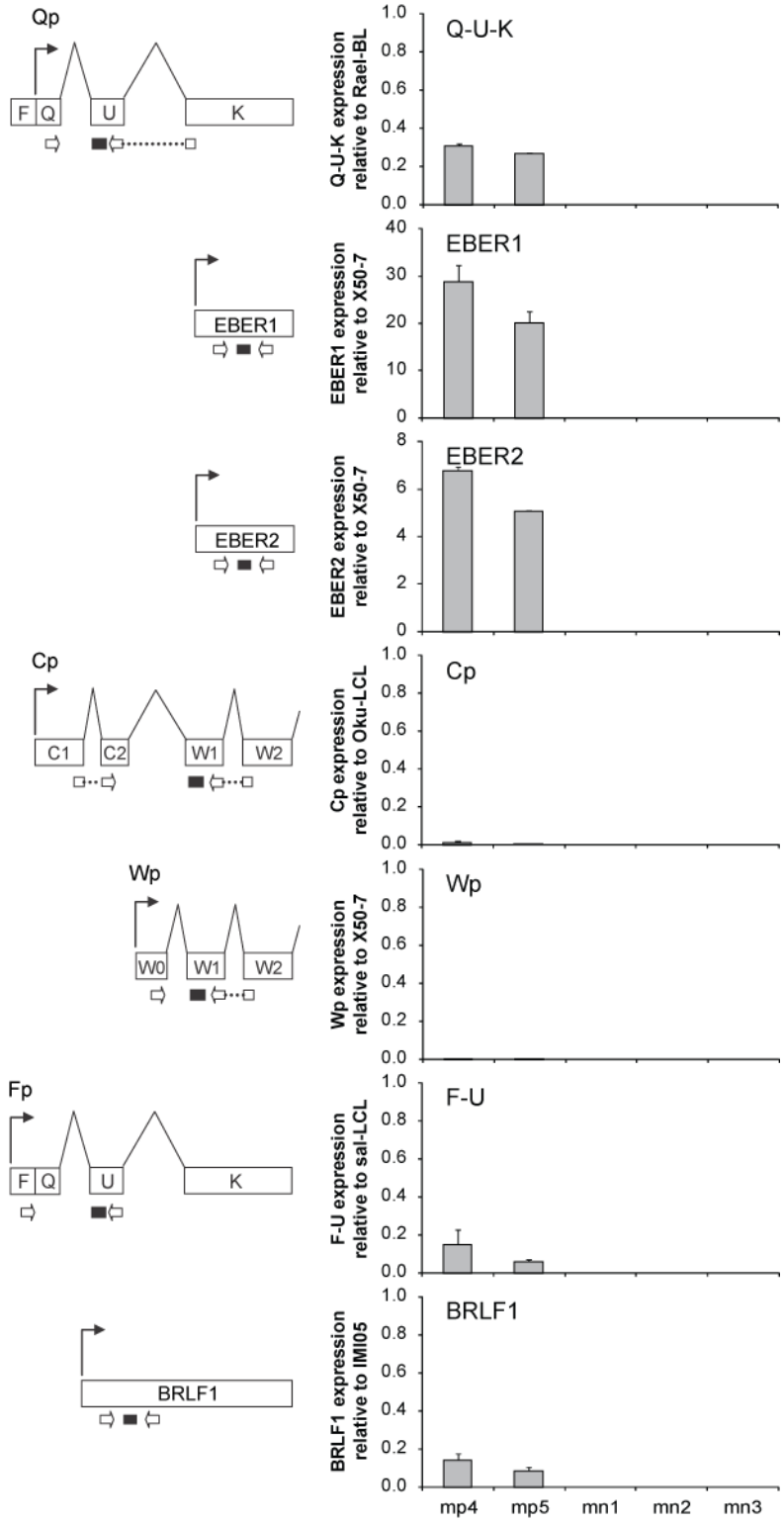


Figure 3.7. EBV gene expression in clones of Mutu-BL as determined by quantitative RT-PCR. Transcription levels were detected using the primer and probe combinations indicated. The hollow arrows represent primers and the black boxes are the TaqMan probes. Transcription levels were measured relative to an appropriate control (assigned a value of 1) and error bars indicate the range between duplicate assays carried out in parallel on the same cDNA sample.

3.3.3 EBV viral latency in clones of Awia-BL

The parental Awia-BL cell line does not conform to the Latency I form of viral gene expression seen in most BL cells, as it contains EBV-positive cells with 3 different forms of viral latency (Kelly et al., 2006). Single cell cloning of Awia-BL generates conventional Latency I clones, Wp restricted clones, novel EBNA2-positive LMP1-negative clones and a small number of EBV-loss clones. For this study, we aimed to compare only paired EBV-positive (Latency I) and EBV-loss clones. These clones were isolated by single cell cloning of Awia-BL and selected by screening for EBV viral load by quantitative DNA-PCR and for expression of EBV latent antigens by western blotting and QRT-PCR.

Single cell cloning of Awia-BL generated 192 clones; of these clones only two were found to have completely lost EBV and these were selected along with 4 EBV-positive clones for further analysis. In the published report of Awia BL latency (Kelly et al., 2006), EBV-positive Latency I clones wp1, wp2, wp3 and wp4 were called a, b, c and d, while the EBV-loss clones wn1 and wn2 were called w and z. They were renamed in this study to reflect the nomenclature of the other EBV-positive and EBV-loss clones.

Initially we extracted genomic DNA from Awia-BL clones and reanalysed EBV viral load using DNA-QPCR (Figure 3.8(A)). Viral load in EBV-positive clones varied from 100 to around 400 EBV genomes per cell, while EBV was undetected in the EBV-loss clones. By western blotting (Figure 3.8(B)), no viral protein expression was observed in EBV-loss clones. EBV-positive clones, however, were found to express EBNA1 at similar levels to X50-7, in the absence of EBNA2 or LMP1, suggesting Latency I viral gene expression.

Next we used QRT-PCR to confirm EBV latent viral transcript expression, investigate viral promoter usage and screen for any activity of the EBV lytic cycle. As shown in Figure 3.9, no EBV latent or lytic transcripts were found in EBV-loss clones, confirming their EBV-negative status. The EBV-positive Awia-BL clones expressed Qp derived Q-U-K transcripts at between 4 and 7 times the level observed in the standard cell line Rael-BL. They also expressed EBER1 transcripts at 40 times the level observed in X50-7 and EBER2 expression of between 2 and 6 times X50-7 levels. Little or no Cp and Wp expression was observed in Awia-BL clones and they were also negative for EBNA2 and LMP1 transcripts (data not shown), confirming that they are truly Latency I BL clones.

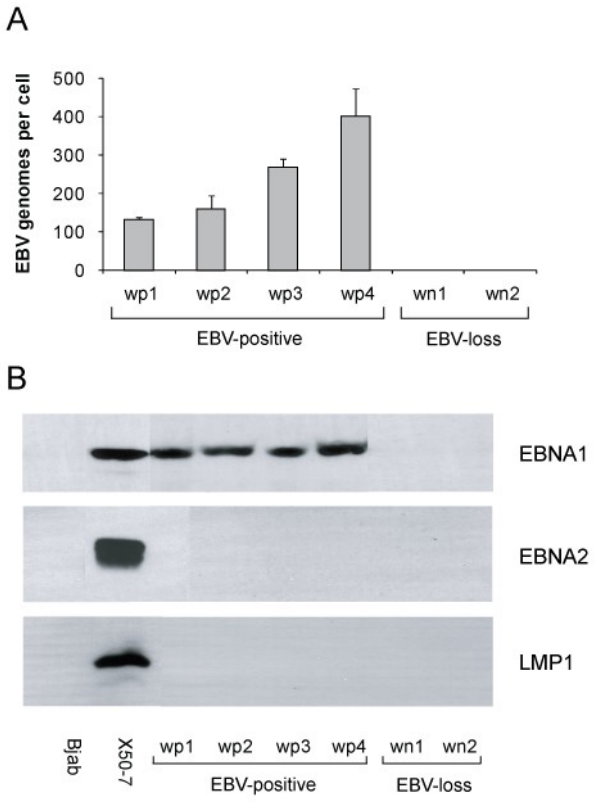


Figure 3.8. Detection of EBV in clones of Awia-BL and investigation of viral latency. (A) Number of EBV genomes per cell as determined by quantitative PCR for the EBV pol gene. Error bars indicate the range between duplicate assays carried out in parallel on the same DNA sample. (B) EBV latent protein expression in Awia-BL clones. Immunoblots were probed with antibodies specific for EBNA1, EBNA2 and LMP1. The positive control is X50-7, an EBV transformed LCL. Bjab is the EBV negative control cell line.

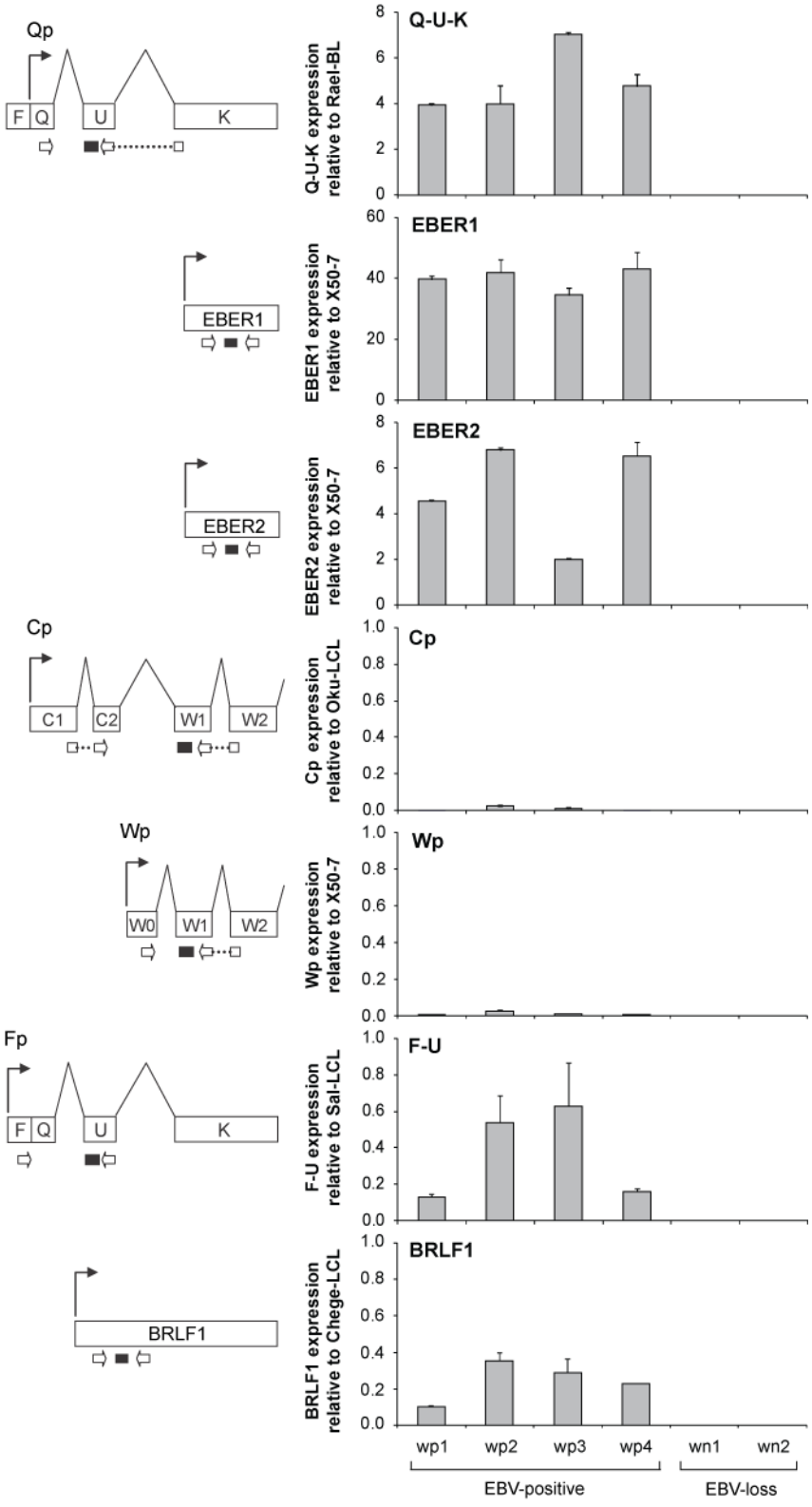


Figure 3.9. EBV gene expression in clones of Awia-BL as determined by quantitative RT-PCR. Transcription levels were detected using the primer and probe combinations indicated. The hollow arrows represent primers and the black boxes are the TaqMan probes. Transcription levels were measured relative to an appropriate control (assigned a value of 1) and error bars indicate the range between duplicate assays carried out in parallel on the same cDNA sample.

When we investigated expression of the EBV lytic cycle in Awia-BL clones, we found low levels of EBV lytic transcripts in all 4 EBV-positive cultures. F-U expression in EBV-positive Awia-BL clones was 10-60% of that observed in the lytic LCL standard (which contains around 2% lytically active cells) and BRLF1 was 10-30% of a similar lytic LCL standard. The low level activity of the EBV lytic cycle may explain the higher viral loads observed in these clones compared to other EBV-positive BL clones; however they still indicated that only a very small percentage of cells are in active lytic cycle.

3.3.4 EBV viral latency in clones of Eli-BL

No EBV-loss clones were generated from single cell cloning of an intermediate passage of Eli-BL. To investigate the effect of long term culture *in vitro* on the propensity of cells to lose EBV, Eli-BL was recloned in late passage, where we found a number of EBV-loss clones. 3 EBV-positive clones, 3 EBV-loss clones and the Eli-BL parental cell line were selected for further analysis.

Figure 3.10(A) shows the average viral load in clones of Eli-BL as determined by QPCR for the EBV pol gene. We found that the EBV viral loads in EBV-positive clones of Eli-BL varied from an average of 10 to nearly 150 EBV genomes per cell. By western blot (Figure 3.10(B)) all EBV-positive clones of Eli-BL expressed EBNA1 in the absence of EBNA2 or LMP1, suggesting Latency I viral gene expression. The 2 EBV-positive clones (ep1 and ep3), which had had lower EBV viral loads, also had lower level expression of EBNA1. EBV was undetectable in EBV-loss clones by quantitative DNA-PCR or western blot.

Next we used QRT-PCR to investigate viral promoter usage, expression of latent viral transcripts and to look for EBV lytic cycle activity (Figure 3.11). As expected, no EBV transcripts were found in EBV-loss Eli-BL clones. Eli-BL and the EBV-positive clone ep2 displayed typical Latency I viral gene expression. Cells had high level expression of Qp derived Q-U-K transcripts, high level expression of EBER1, and expressed levels of EBER2 of around 40% of the level observed in the LCL control cell line, X50-7. Cp and Wp initiated transcripts were almost absent in these cell lines and there was little or no expression of EBNA2 or LMP1 transcripts (data not shown). As seen in EBV-positive clones of other BL cell lines, the presence of F-U and BRLF1 transcripts indicated a small number of cells in these cultures were in lytic cycle.

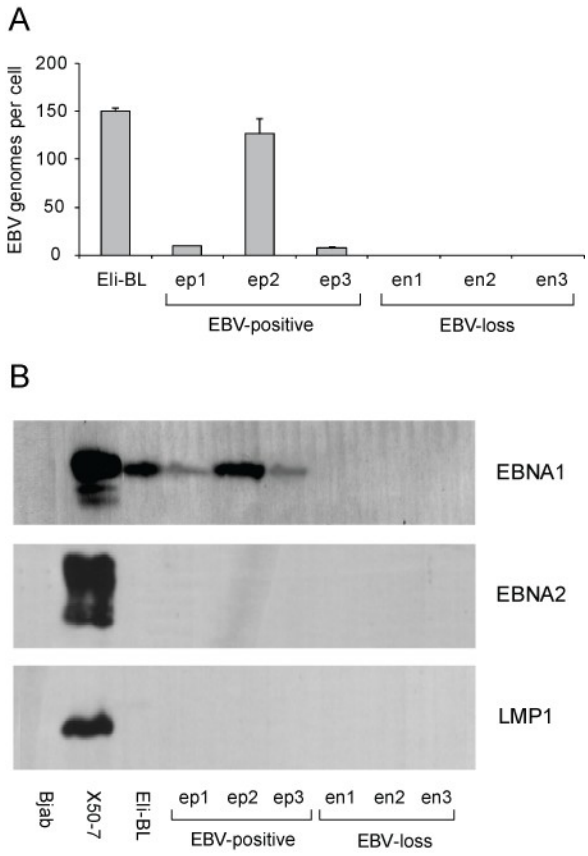


Figure 3.10. Detection of EBV in clones of Eli-BL and investigation of viral latency. (A) Number of EBV genomes per cell as determined by quantitative PCR for the EBV pol gene. Error bars indicate the range between duplicate assays carried out in parallel on the same DNA sample. (B) EBV latent protein expression in Eli-BL clones. Immunoblots were probed with antibodies specific for EBNA1, EBNA2 and LMP1. The positive control is X50-7, an EBV transformed LCL. Bjab is the EBV negative control cell line.

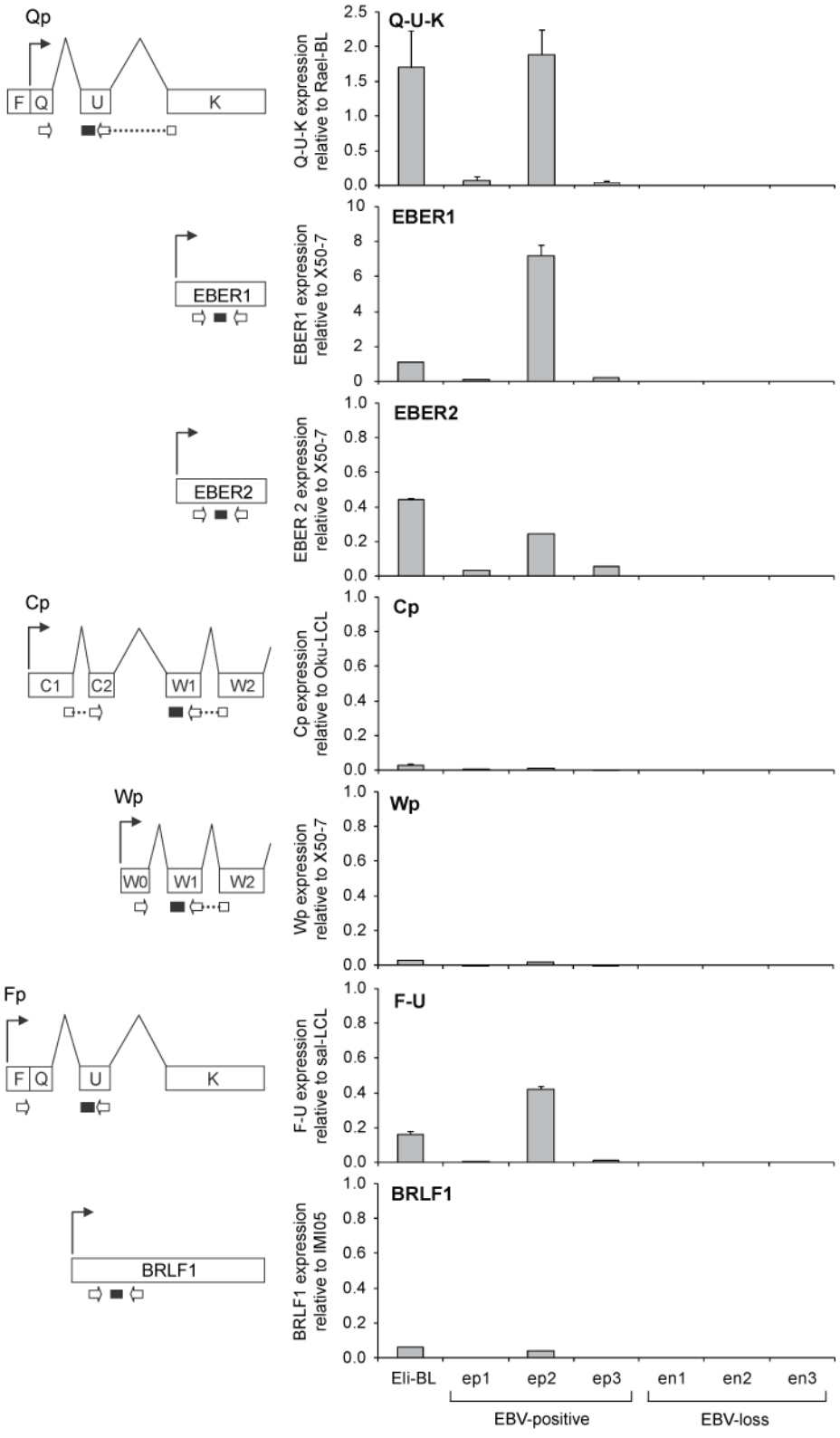


Figure 3.11. EBV gene expression in clones of Eli-BL determined by quantitative RT-PCR. Transcription levels were detected using the primer and probe combinations indicated. The hollow arrows represent primers and the black boxes are the TaqMan probes. Transcription levels were measured relative to an appropriate control (assigned a value of 1) and error bars indicate the range between duplicate assays carried out in parallel on the same cDNA sample.

Interestingly, the other 2 EBV-positive clones (ep1 and ep3) did not appear to have normal latent viral gene expression. Analysis by QRT-PCR revealed very low expression of all the latent viral transcripts. This, combined with the lower viral load and reduced EBNA1 expression seen in Figure 3.10(B), indicates that cells from these EBV-positive clones had continued to lose EBV during cell culture. For this reason, these clones were not included in further study and the effect of EBV on cell phenotype in Eli-BL was determined using an intermediate passage of the Eli-BL parental cell line and the Latency I clone, ep2.

3.3.5 EBV viral latency in clones of Kem-BL

As observed in Eli-BL, no EBV-loss clones were generated from single cell cloning of an intermediate passage of Kem-BL. In an attempt to generate EBV-loss clones, we single cell cloned a culture of Kem-BL which had been cultured *in vitro* for approximately 120 passages. From this second cloning experiment we isolated 44 single cell clones, of which 43 were EBV-positive. The remaining clone had an average viral load of less than 0.1 genomes per cell, strongly suggesting that it contained a mixture of EBV-positive and EBV-loss cells. This mixed Kem-BL clone was recloned and the panel of subclones generated was screened for the presence of EBV by QPCR (data not shown). Most subclones were completely EBV-negative and those that were EBV-positive had average viral loads of less than 1 EBV genome per cell. For this reason we selected 3 EBV-positive clones from the 43 clones generated from the cloning of late passage Kem-BL to further analyse along with 3 EBV-loss clones. We determined EBV viral latency by western blotting and QRT-PCR and investigated activity of the EBV lytic cycle by QRT-PCR.

When we repeated the EBV viral load QPCR on the 6 selected clones (Figure 3.12(A)), we found that viral load in EBV-positive clones varied within an average of 10-40 EBV genomes per cell, while EBV was undetectable in EBV-loss clones. By western blot (Figure 3.12(B)), we found no expression of EBV proteins in EBV-loss clones. EBV positive clones expressed EBNA1 in the absence of EBNA2 or LMP1, indicating that cells retained the restricted Latency I viral gene expression seen in the parental Kem-BL cell line.

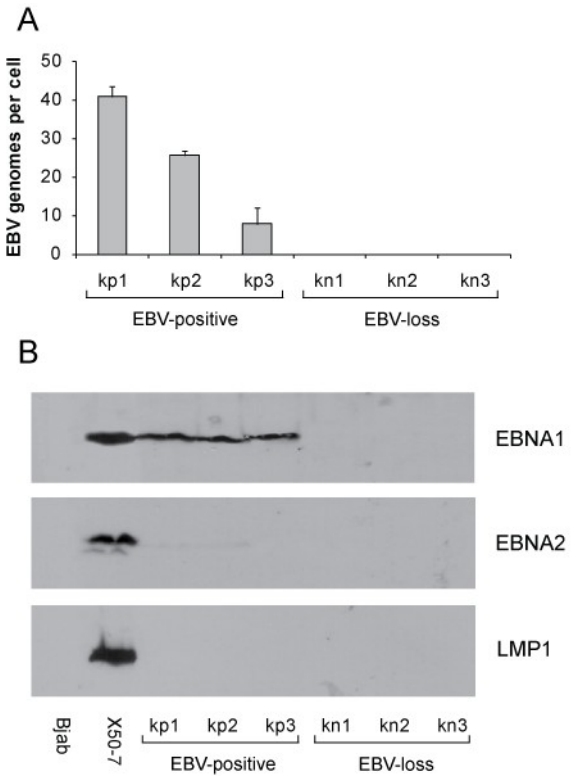


Figure 3.12. Detection of EBV in clones of Kem-BL and investigation of viral latency. (A) Number of EBV genomes per cell as determined by quantitative PCR for the EBV pol gene. Error bars indicate the range between duplicate assays carried out in parallel on the same DNA sample. (B) EBV latent protein expression in Kem-BL clones. Immunoblots were probed with antibodies specific for EBNA1, EBNA2 and LMP1. The positive control is X50-7, an EBV transformed LCL. Bjab is the EBV negative control cell line.

To confirm this, we used QRT-PCR to investigate viral promoter usage, expression of latent viral transcripts and to look for EBV lytic cycle activity (Figure 3.13). Qp activity was confirmed in EBV-positive Kem-BL clones by expression of Q-U-K transcripts at similar levels to the control cell line, Rael-BL. They expressed EBER1 and EBER2 transcripts several fold higher than the standard cell line, X50-7, but in contrast to the other cell lines investigated, EBV-positive Kem BL clones expressed similar levels of EBER1 and EBER2 transcripts. Cp and Wp initiated transcripts were almost absent in EBV-positive clones and there was little or no expression of EBNA2 or LMP1 transcripts (data not shown). Qp activity in the absence of Cp or Wp transcripts confirms restricted Latency I viral gene expression in EBV-positive Kem-BL clones. As seen in EBV-positive clones of other BL cell lines, a low level of F-U and BRLF1 transcripts indicated a small number of cells in EBV-positive clones to be in lytic cycle. Overall QRT-PCR analysis revealed that EBV-positive Kem-BL clones display the typical Latency I viral gene expression seen in BL tumours and in EBV-positive clones from other BL lines.

In conclusion, we have generated matched EBV-positive and EBV-loss clones from 4 eBL cell lines and from the sporadic BL cell line, Akata-BL. With the exception of Latency III Mutu-BL clones all the EBV-positive clones remained in Latency I; thus these cell lines provide an excellent model for the investigation of the effect of Latency I gene expression in BL cells.

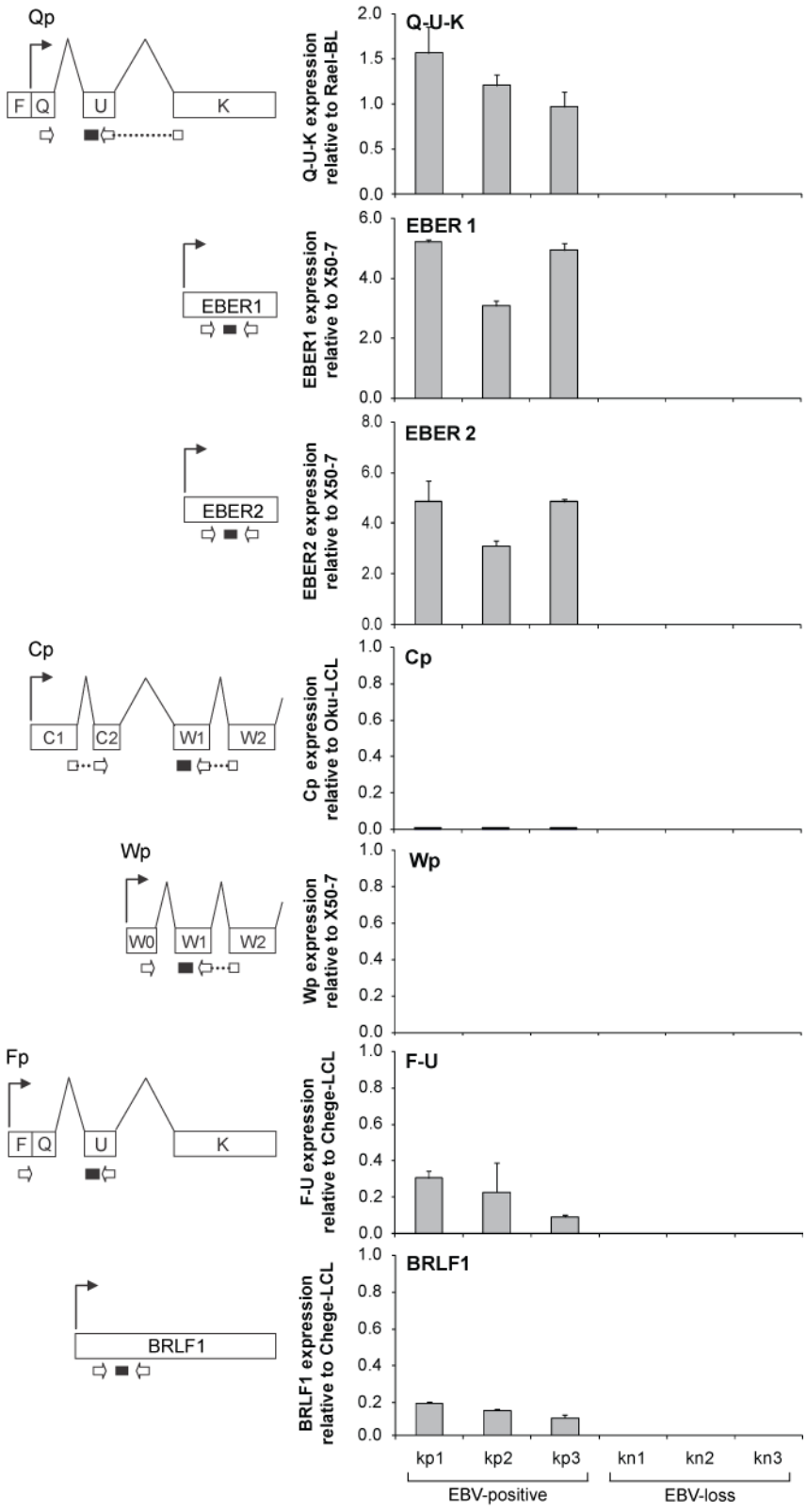


Figure 3.13. EBV gene expression in clones of Kem-BL as determined by quantitative RT-PCR. Transcription levels were detected using the primer and probe combinations indicated. The hollow arrows represent primers and the black boxes are the TaqMan probes. Transcription levels were measured relative to an appropriate control (assigned a value of 1) and error bars indicate the range between duplicate assays carried out in parallel on the same cDNA sample.

3.4 Apoptosis resistance in EBV-positive and EBV-loss clones

It has been previously reported that loss of EBV from Akata-BL cells increased their sensitivity to apoptosis (Komano et al., 1998). To test this observation, we compared apoptosis sensitivity in the newly generated panel of EBV-positive and EBV-loss clones.

Figure 3.14(A) shows a schematic representation of the method used to induce and detect cell death in BL cells. Following exposure to apoptotic stimulus, live, apoptotic and necrotic cells were detected by dual staining with the fluorescent DNA binding dyes, Syto 16 and propidium iodide (PI). As shown in Figure 3.14(B), analysis by flow cytometry was used to construct a two-dimensional dot plot of Syto 16 staining (y-axis) versus PI staining (x-axis). Syto 16 is actively pumped into viable cells and PI preferentially enters cells with compromised cell membranes, so stains necrotic cells. This method allows the discrimination of live cells (Syto 16 positive, PI negative), apoptotic cells (Syto 16 negative, PI negative) and necrotic cells (Syto 16 negative, PI positive).

3.4.1 Resistance to ionomycin induced apoptosis in EBV-positive and EBV-loss clones of Akata-BL

We initially induced apoptosis in BL clones by treatment with the calcium ionophore, ionomycin. Levels of intracellular Ca^{2+} ions exceeding the natural buffering capacity of the cytosol are a well known trigger of apoptosis (Orrenius et al., 2003; Berridge et al., 1998; McConkey and Orrenius, 1997). To effectively highlight any difference in apoptosis resistance between EBV-positive and EBV-loss clones, we initially sought to determine an ionomycin concentration that induced around 60% cell death in the most sensitive Akata-BL clones. Cells were treated for 48 hours with 0.25µg/mL, 0.5µg/mL or 1µg/mL ionomycin, stained with Syto 16 and PI and analysed by flow cytometry.

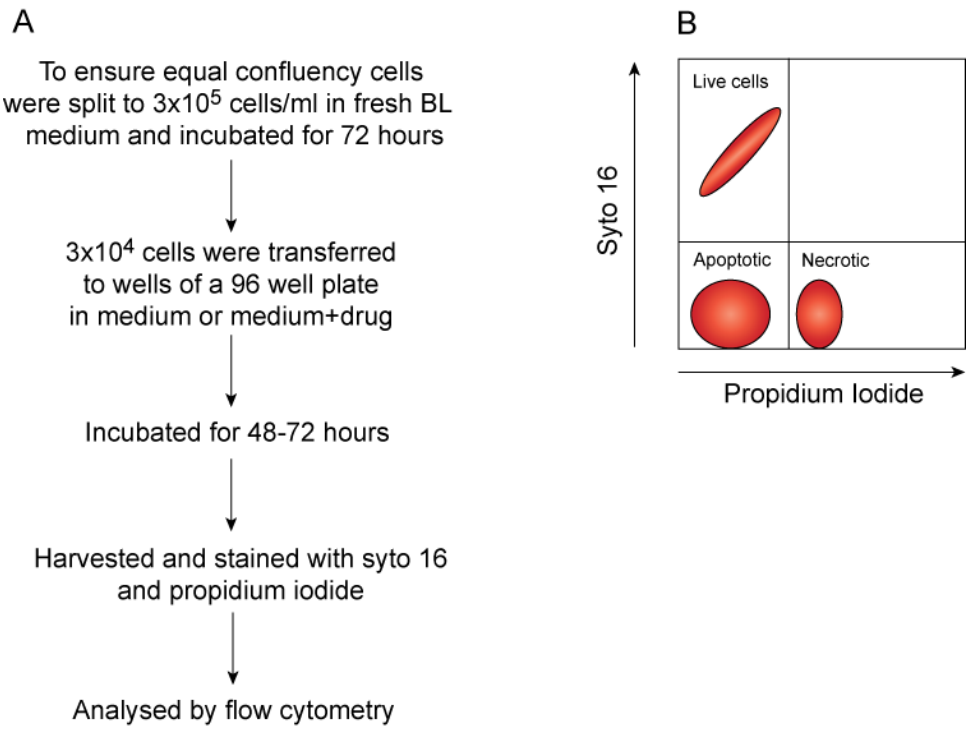


Figure 3.14. Measurement of apoptosis resistance in BL cells. (A) Method used to induce and measure cell death in BL cells. (B) Schematic representation of a 2 dimensional dot plot showing fluorescence signals generated by flow cytometric analysis of Syto 16 and propidium iodide stained cells. This method allows the discrimination of live cells (Syto 16 positive, PI negative), apoptotic cells (Syto 16 negative, PI negative) and necrotic cells (Syto 16 negative, PI positive).

Figure 3.15(A) shows flow cytometric data from an EBV-positive clone (ap1) and an EBV-loss clone (an1). Increasing doses of ionomycin induced escalating levels of cell death in both Akata-BL clones. Nearly all cells induced into cell death by ionomycin died by apoptosis. Interestingly, at all 3 ionomycin concentrations, induced cell death was greater in the EBV-loss clone (an1) than in the EBV-positive clone (ap1). Next, we expanded analysis of ionomycin induced cell death to include the remaining EBV-positive and EBV-loss clones and the parental Akata-BL cell line. Percentage induced cell death in Akata-BL clones after treatment with 0.25µg/mL, 0.5µg/mL and 1µg/mL ionomycin is summarised into bar charts in Figure 3.15(B). Cell death in each clone is divided into the percentage induced apoptosis and percentage induced necrosis. All three concentrations of ionomycin induced more cell death in EBV-loss clones when compared to EBV-positive clones or the Akata-BL parental cell line. The greatest difference in percentage induced cell death between EBV-positive and EBV-loss clones was found at 0.5µg/mL ionomycin. This concentration of ionomycin induced approximately 70% of cells from EBV-loss clones into cell death compared to only 40% of EBV-positive cells. It is clear from Figure 3.15(B) that increased cell death in EBV-loss clones of Akata-BL was due to increased sensitivity to apoptosis. There appeared to be no difference in the sensitivity of cells to ionomycin induced necrosis. This experiment was repeated several times and we found similar results on each occasion.

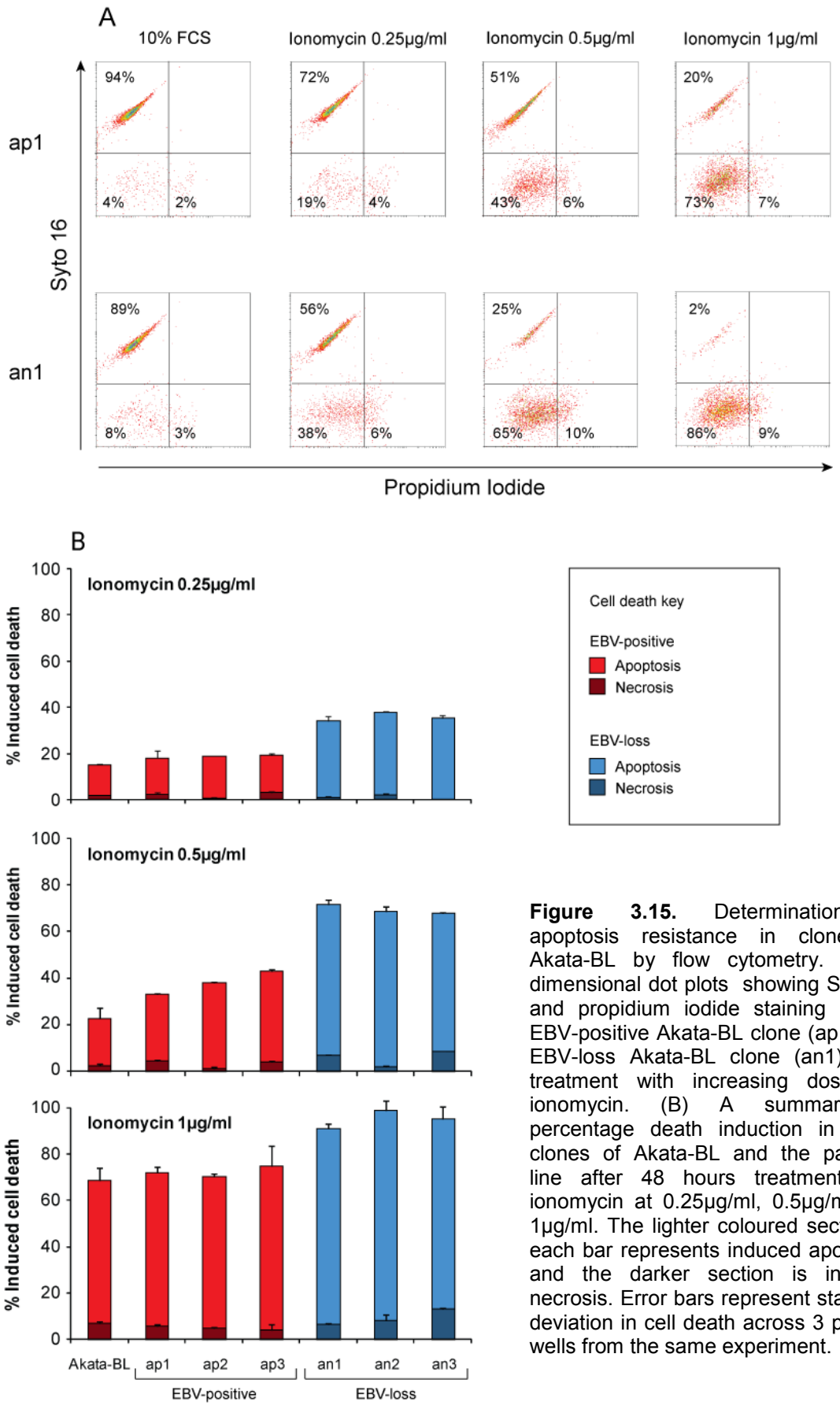


Figure 3.15. Determination of apoptosis resistance in clones of Akata-BL by flow cytometry. (A) 2 dimensional dot plots showing Syto 16 and propidium iodide staining in the EBV-positive Akata-BL clone (ap1) and EBV-loss Akata-BL clone (an1) after treatment with increasing doses of ionomycin. (B) A summary of percentage death induction in all 6 clones of Akata-BL and the parental line after 48 hours treatment with ionomycin at 0.25µg/ml, 0.5µg/ml and 1µg/ml. The lighter coloured section of each bar represents induced apoptosis and the darker section is induced necrosis. Error bars represent standard deviation in cell death across 3 parallel wells from the same experiment.

3.4.2 Resistance to ionomycin induced apoptosis in EBV-positive and EBV-loss clones of eBL cell lines

To determine the contribution of EBV to apoptosis resistance in endemic BL cell lines, ionomycin was used to induce cell death in representative EBV-positive and EBV-loss clones of Mutu-BL, Awia-BL, Eli-BL and Kem-BL. EBV-positive Latency I clones and EBV-loss clones of Mutu-BL were analysed alongside clones expressing the highly protective Latency III growth transforming programme. As with clones of Akata-BL, ionomycin was initially used to induce cell death at a range of different concentrations. An ionomycin concentration was selected for each cell line which induced around 60% cell death in the most sensitive clones and the results are shown in Figure 3.16. Clones of Mutu-BL, Eli-BL and Kem-BL required 0.5µg/mL of ionomycin to induce sufficient cell death; clones of Awia-BL appeared to be more sensitive and only 0.25µg/mL ionomycin was required. Ionomycin induced cell death in all BL cell lines was predominantly by apoptosis.

The percentage induced cell death in EBV-loss clones of Akata-BL was slightly less than 2-fold greater than in EBV-positive clones. We found that loss of EBV from endemic BL lines caused a much greater increase in apoptosis sensitivity. 0.5µg/mL ionomycin induced less than 20% cell death in EBV-positive (Latency I) Mutu-BL clones mp1, mp2 and mp3, but caused nearly 80% cell death in EBV-loss clones. The difference in apoptosis sensitivity between EBV-positive Latency I and Latency III clones could only be seen at greater ionomycin concentrations (data not shown). A similar difference in apoptosis resistance between Latency I and EBV-loss clones was also observed when mp1, mp2 and mp3 were substituted for the alternative Mutu-BL clones, mp4 and mp5, again demonstrating that mp4 and mp5 represent typical Latency I Mutu-BL clones. EBV-loss clones of Awia-BL, Eli-BL and Kem-BL were again consistently more sensitive to apoptosis than EBV-positive clones and the difference in sensitivity was noticeably greater than in clones of Akata-BL. These experiments were repeated several times and we found similar results on each occasion.

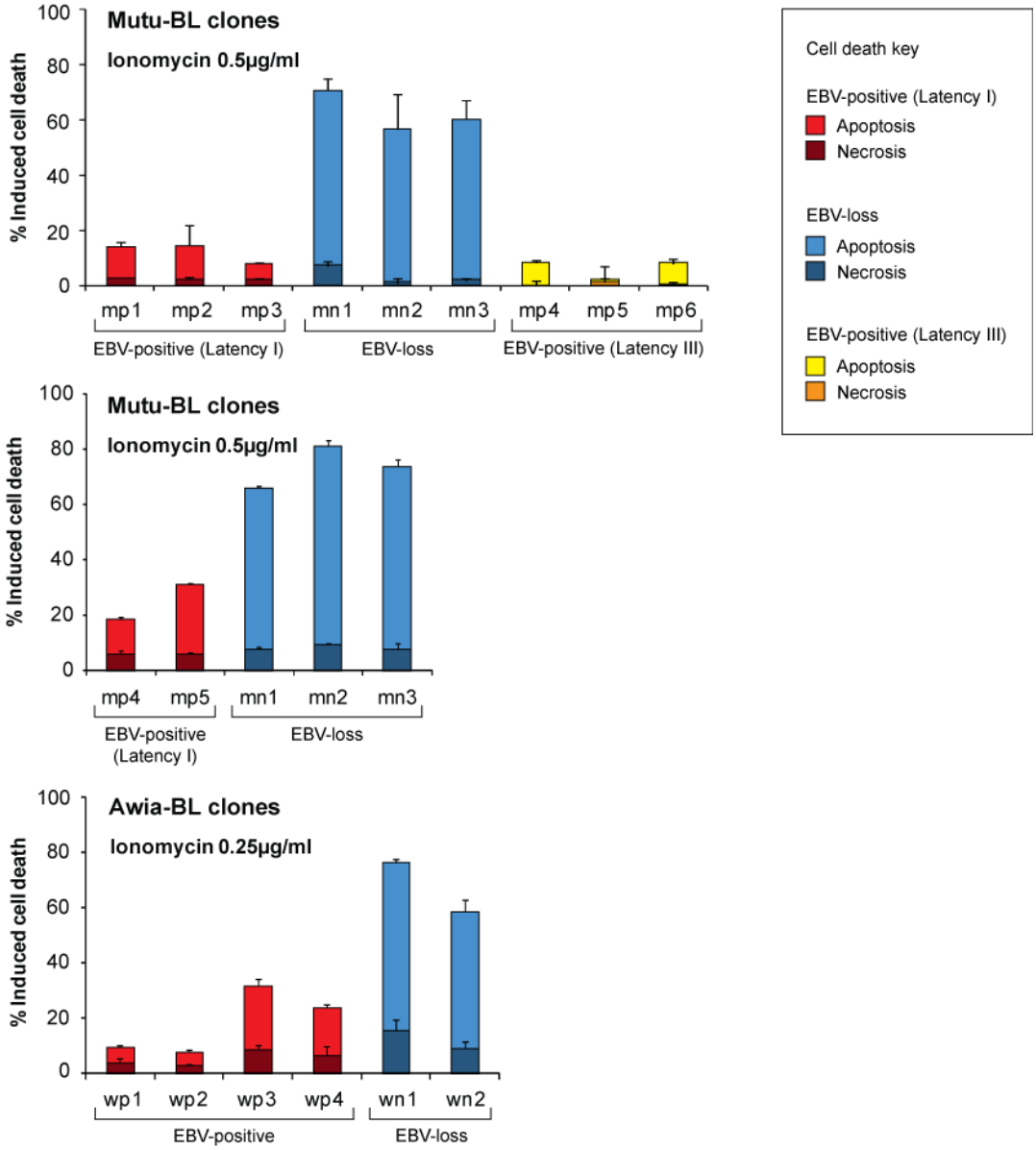


Figure 3.16. Percentage induced cell death in EBV-positive and EBV-loss clones of Mutu-BL and Awia-BL after 48 hour treatment with ionomycin. Apoptosis and necrosis were determined by Syto 16 and propidium iodide staining as shown in Figure 3.14. The lighter coloured section of each bar represents induced apoptosis and the darker section is induced necrosis. Error bars represent standard deviation in cell death across 3 parallel wells from the same experiment.

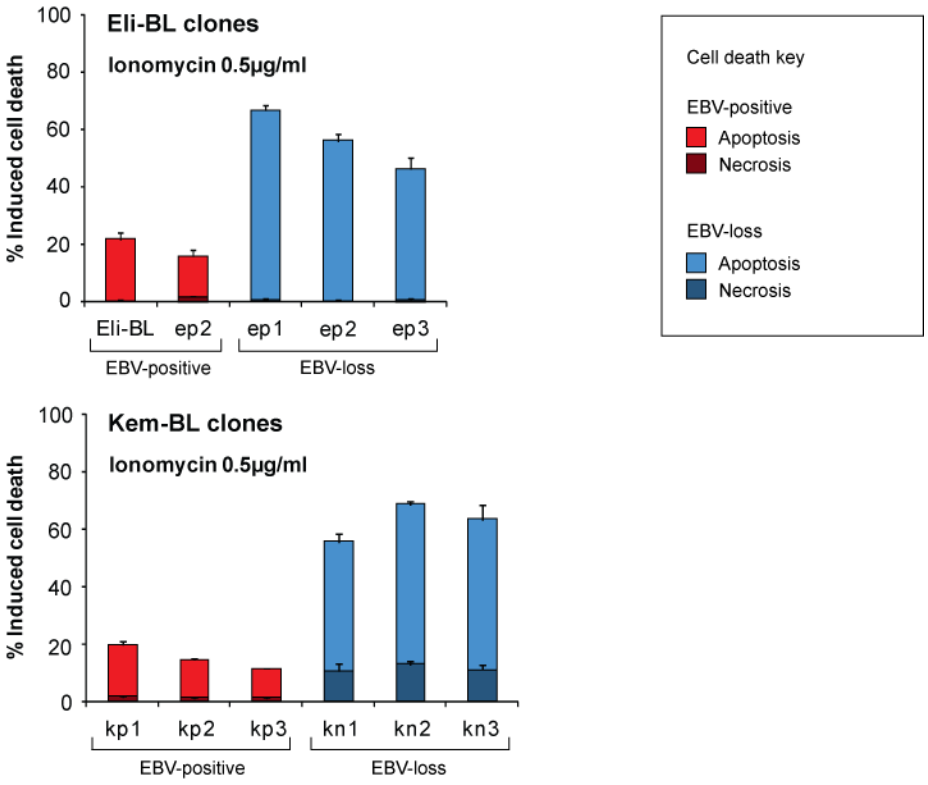


Figure 3.16 (continued). Percentage induced cell death in EBV-positive and EBV-loss clones of Eli-BL and Kem-BL after 48 hour treatment with ionomycin. Apoptosis and necrosis were determined by Syto 16 and propidium iodide staining as shown in Figure 3.14. The lighter coloured section of each bar represents induced apoptosis and the darker section is induced necrosis. Error bars represent standard deviation in cell death across 3 parallel wells from the same experiment.

3.4.3 Resistance to anti-IgM induced apoptosis in EBV-positive and EBV-loss clones of eBL cell lines

Anti-IgM cross-linking induces apoptosis in surface IgM positive cell lines through generation of inositol trisphosphate (InsP3), which causes the release of Ca^{2+} from intracellular stores and subsequent activation of caspases, 2, 3, and 9 (Chen et al., 1999b; Gold and DeFranco, 1987). Hence we investigated the effect of this second apoptosis trigger in EBV-positive and EBV-loss clones of the surface IgM positive cell lines Mutu-BL, Awia-BL and Kem-BL. Cells were treated with a range of anti-IgM concentrations for 72 hours. As shown in Figure 3.17, the optimum anti-IgM concentration for induction of cell death in clones of Mutu-BL, Awia-BL and Kem-BL was found to be 5µg/mL. Cell death was predominantly by apoptosis, although there was slightly more necrosis than was observed after ionomycin treatment.

5µg/mL anti-IgM induced approximately 40% cell death in the EBV positive Latency I Mutu-BL clones, mp1, mp2 and mp3. However cell death in EBV-loss clones was nearly 2-fold higher, demonstrating that EBV-mediated apoptosis resistance is not limited to protection from ionomycin. 5µg/mL anti-IgM induced only around 20% of Latency III Mutu-BL into cell death. Once again EBV-positive clones mp4 and mp5 demonstrated the same increased resistance to apoptosis as clones mp1, mp2 and mp3. Thus, Latency I gene expression provides a significant degree of resistance, but is unable to protect cells as comprehensively as a full Latency III infection.

We found that loss of EBV from Awia-BL and Kem-BL cells also increased anti-IgM induced cell death by around 2-fold. As we observed after treatment with ionomycin, differences in anti-IgM induced cell death were due entirely to increased apoptosis in EBV-loss clones. We found no difference in the level of induced necrosis between EBV-positive Latency I clones, EBV-loss clones or EBV-positive Latency III clones.

In conclusion, we found that loss of EBV from BL cells was associated with an increased sensitivity to apoptosis in all 5 BL cell lines that yielded EBV-loss clones. The effect of EBV-loss on apoptosis sensitivity appeared to be greater in endemic BL lines compared to the sporadic BL cell line Akata-BL.

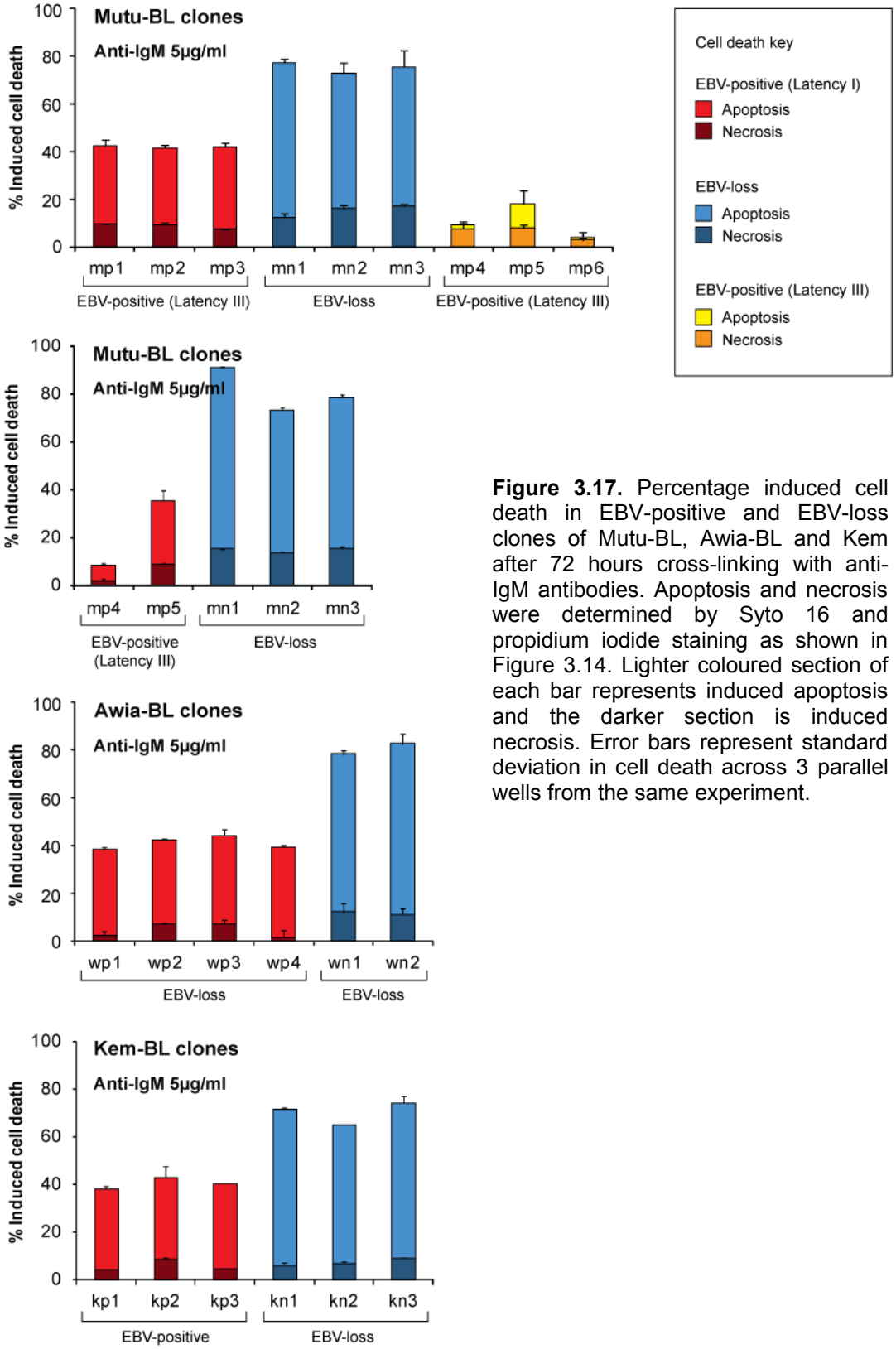


Figure 3.17. Percentage induced cell death in EBV-positive and EBV-loss clones of Mutu-BL, Awia-BL and Kem after 72 hours cross-linking with anti-IgM antibodies. Apoptosis and necrosis were determined by Syto 16 and propidium iodide staining as shown in Figure 3.14. Lighter coloured section of each bar represents induced apoptosis and the darker section is induced necrosis. Error bars represent standard deviation in cell death across 3 parallel wells from the same experiment.

3.4.4 Resistance to cytotoxic drugs

Both ionomycin and anti-IgM cross-linking induce apoptosis through release of intracellular calcium. To examine if loss of the EBV genome increased sensitivity to other inducers of apoptosis we tested the effects of 5 cytotoxic drugs, namely roscovitine, etoposide, nocodazole, rotenone and cisplatin. We selected these drugs because of their well documented induction of apoptosis in BL cells (Wade and Allday, 2000; Reeves et al., 2007; Anderton et al., 2008). Roscovitine is a selective inhibitor of cyclin-dependent kinases (Cdks). Etoposide induces single and double strand DNA breaks and inhibits synthesis of the oncoprotein MDM2. Nocodazole induces apoptosis by disrupting the formation of microtubules and thus arresting cells in G_2/M phase. Rotenone interferes with mitochondrial electron transport, while cisplatin induces apoptosis by DNA cross-linking. Cell death was induced in clones of Akata-BL, Awia-BL and Mutu-BL using a number of different concentrations of each drug and induced apoptosis and necrosis were determined with Syto 16 and propidium iodide staining.

The results of cytotoxic drug induced cell death are shown in Figure 3.18. In each cell line, cell death is shown at the concentration of cytotoxic drug which induced closest to 60% cell death in the most sensitive clones. As a positive control for EBV-mediated protection from apoptosis, aliquots of cells were also treated with ionomycin as described above.

Roscovitine induced cell death in clones of Akata-BL at 50μM and in clones of Awia-BL and Mutu-BL at 20μM. Cell death was mainly by apoptosis, although drug induced necrosis was also detected. EBV-loss clones of Akata-BL, Awia-BL and Mutu-BL all appeared to be more sensitive to the action of roscovitine than matched EBV-positive clones, although the difference was not as striking as observed after treatment with ionomycin or anti-IgM.

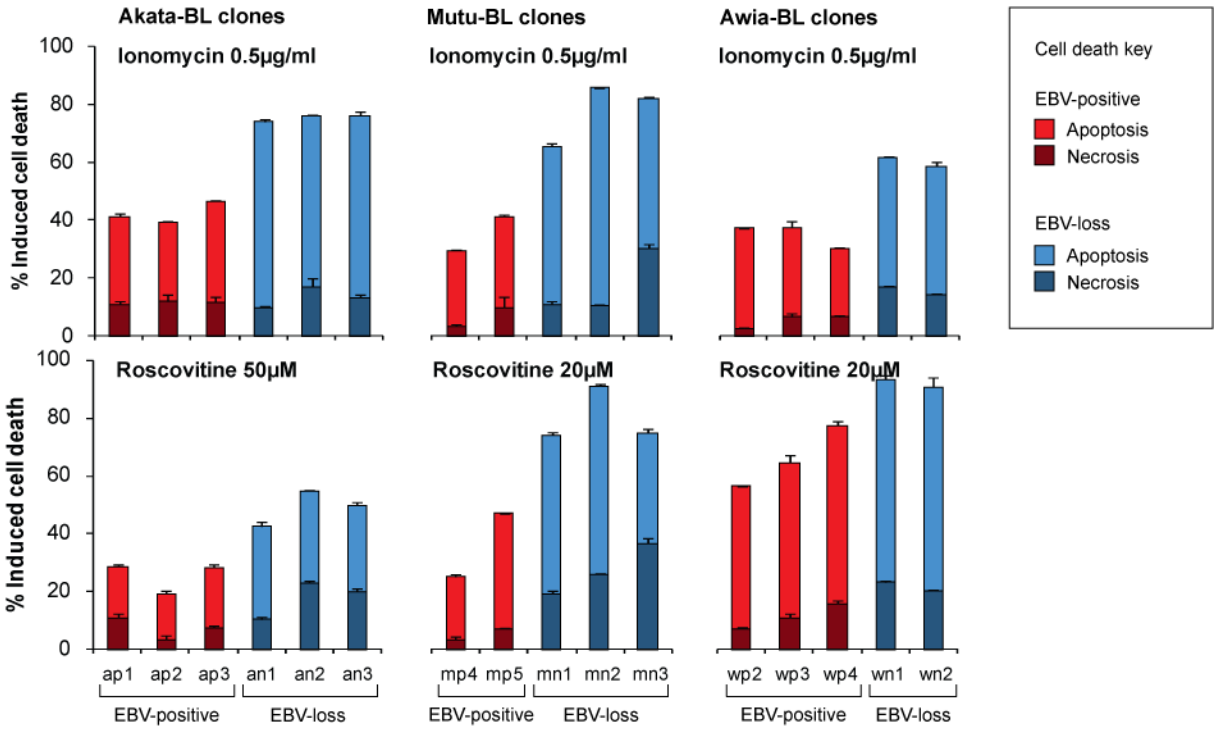


Figure 3.18. Sensitivity of EBV-positive and EBV-loss clones of Akata-BL, Awia-BL and Mutu-BL to ionomycin and roscovitine. Cell death was determined by Syto 16 and propidium iodide staining (Figure 3.14). The lighter coloured section of each bar represents induced apoptosis and the darker section is induced necrosis. Error bars represent standard deviation in cell death across 3 independent wells.

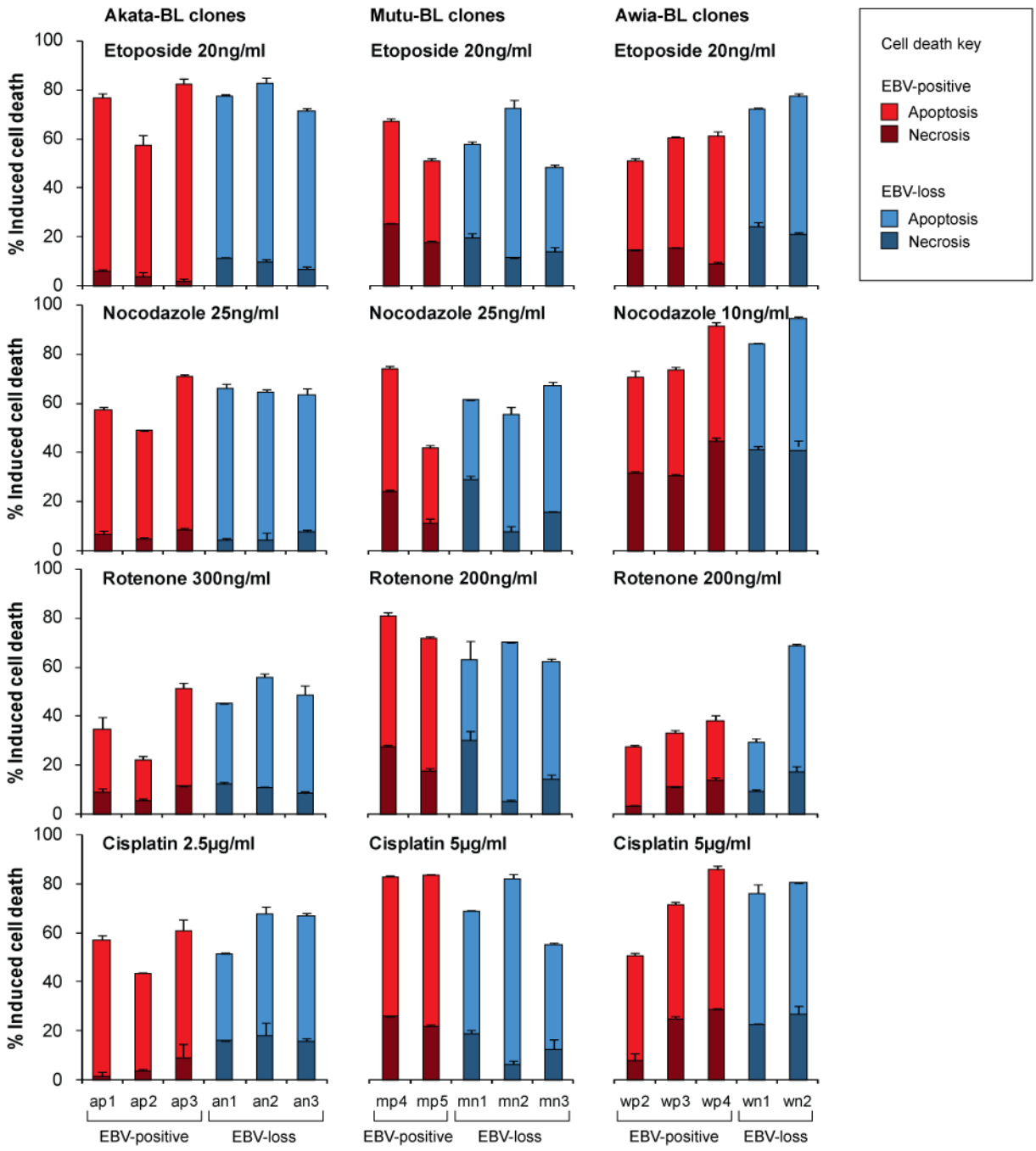


Figure 3.18 (continued) Sensitivity of EBV-positive and EBV-loss clones of Akata-BL, Awia-BL and Mutu-BL to etoposide, nocodazole, rotenone and cisplatin. Cell death was determined by Syto 16 and propidium iodide staining (Figure 3.14). The lighter coloured section of each bar represents induced apoptosis and the darker section is induced necrosis. Error bars represent standard deviation in cell death across 3 independent wells.

We also induced cell death with etoposide, nocodazole, rotenone and cisplatin at a range of different concentrations. We found that cell death was predominantly by apoptosis; however there was no difference in the sensitivity of EBV-positive and EBV-loss clones to these drugs. Thus EBV appears able to protect cells from apoptosis induced by calcium efflux and inhibition of Cdks, but is unable to protect cells from the effect of direct DNA damage, inhibition of microtubule formation or disruption of mitochondrial electron transport.

By studying the published mechanisms of apoptosis induction for each of these drugs we discovered that all the drugs from which EBV was unable to protect BL cells (etoposide, nocodazole, rotenone and cisplatin) ultimately induce apoptosis through the p53 pathway, while all the drugs from which EBV was able to protect BL cells were able to signal apoptosis through p53-independent mechanisms. To investigate this phenomenon, we examined p53 and MDM2 protein levels in EBV-positive and EBV-loss clones of Akata-BL, Mutu-BL and Awia-BL and compared this data to previous sequencing work carried out in our lab on the p53 gene in various BL cell lines. As shown in Figure 3.19, Akata-BL clones are completely null for p53, while low level p53 expression can be observed in Awia-BL clones and higher levels can be detected in clones of Mutu-BL. Interestingly however, both Mutu-BL and Awia-BL have mutations of p53 leading to expression of a non-functional protein. All the BL clones expressed the anti-apoptotic MDM2 protein and the expression of p53 and MDM2 appears to be unaffected by EBV status. The possible exception is a slight increase in p53 expression in EBV-loss clones of Awia-BL, but as this is mutated non-functional p53 it is unlikely to affect apoptosis resistance.

It is interesting that cytotoxic drugs which act primarily through p53-dependent pathways are able to induce cell death in BL clones with crippling p53 mutations. Disruption of this pathway in BL cells may be the reason why EBV is unable to protect cells from drugs which primarily induce apoptosis through p53. Although this is an interesting observation, more work is required to determine its relevance for BL pathogenesis. Unfortunately, despite that fact that this work is presented in here it was not carried out until late in the PhD study so could not be investigated further.

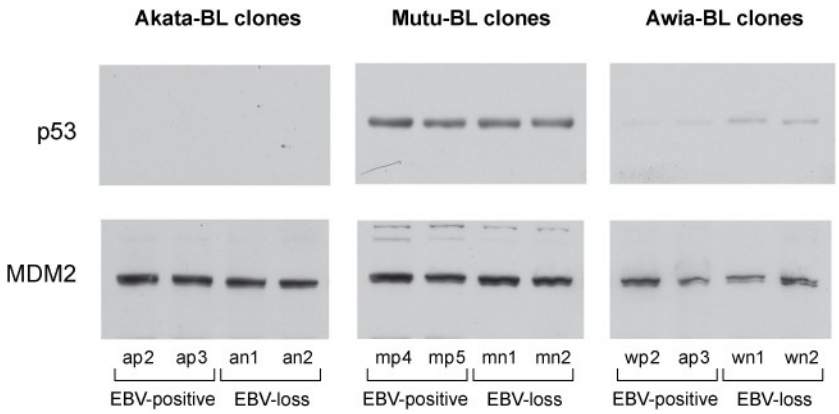


Figure 3.19. Expression of p53 and MDM2 in BL clones. Immunoblots were probed with specific antibodies against p53 and MDM2 in EBV-positive and EBV-loss clones of Akata-BL, Mutu-BL and Awia-BL.

3.5 Growth capacity and serum dependence of BL clones

Loss of EBV has been reported to render Akata-BL cells unable to grow in medium supplemented with only 0.1% foetal calf serum (FCS) (Shimizu et al., 1994). We therefore investigated the growth ability under optimal conditions (10% FCS) and survival ability in low serum (0.1% FCS), of paired EBV-positive and EBV-loss clones of Akata-BL, Mutu-BL, Awia-BL and Eli-BL. Cells were split to a density of $3x10^5$ cell/mL 3 days before the start of the experiment to ensure equal confluency, then transferred to BL medium containing 10% or 0.1% FCS. Cell viability was then monitored by trypan blue exclusion for the next 6-8 days or until all the cells within the culture were dead and the experiment was carried out at least twice for each cell line investigated.

Growth capacity under optimal growth conditions and survival in low serum of 3 EBV-positive and 3 EBV-loss clones of Akata-BL is shown in Figure 3.20. In 10% FCS, Akata-BL cells followed the classical eukaryotic growth curve. A one day lag phase was followed by two days of exponential cell growth. The number of viable cells then remained stationary for two further days before cells began to die. EBV-positive clones of Akata-BL reached a maximum cell density of $1.0x10^6$ cells/mL in experiment 1 and $1.2x10^6$ cells/mL in experiment 2. Cell density of EBV-loss clones peaked at $0.9x10^6$ and $1.0x10^6$ cells/mL in experiment 1 and 2. This would suggest a small proliferative advantage for cells harbouring EBV. However the general variability between the growth capacities of different Akata-BL clones was such that no firm conclusions can be drawn on a possible growth advantage for EBV-positive cells. In 0.1% FCS both EBV-positive and EBV-loss clones of Akata-BL proliferated for the first 2-3 days of the experiment. After three days cells began to die rapidly and no viable cells remained within the culture after 6 days. Loss of EBV did not appear to affect the ability of Akata-BL clones to survive in low serum.

Growth of Mutu-BL clones under optimum conditions followed the same general pattern as in clones of Akata-BL (Figure 3.21). EBV-positive clones appeared to proliferate slightly more quickly during the first three days of the experiment. However the difference was again small compared to the general variability in growth capacity of Mutu-BL clones and both EBV-positive and EBV-loss Mutu-BL clones eventually reached the same maximum cell density of around $2.5x10^6$ cells/mL. In 0.1% FCS, both EBV-positive and EBV-loss Mutu-BL clones showed very little proliferation. But in contrast to Akata-

BL, loss of EBV from Mutu-BL cells resulted in a pronounced decrease in survival ability. No EBV-loss clones survived for more than three days in 0.1% FCS, whereas EBV-positive clones remained viable for up to 6 days. This phenotype was observed in both the original EBV-positive Mutu-BL clones (mp1, mp2 and mp3) and their replacements (mp4 and mp5).

As shown in Figure 3.22, clones of Awia BL proliferated slightly slower than clones of Akata-BL or Mutu-BL. We therefore extended this growth assay for a further two days. Awia-BL clones reached a maximum cell density after 4-5 days in culture of $2.5x10^6$ cells/mL. Under optimal conditions there appeared to be little difference in growth capacity between EBV-positive and EBV-loss clones. The viability of cultures of both EBV-positive and EBV-loss clones dropped very rapidly in 0.1% FCS so the experiment was repeated in 1% serum. With the exception of a single EBV-positive clone in one experiment, EBV-positive clones of Awia-BL appeared to have a small increase in survival ability in 1% FCS compared to EBV-loss clones.

Finally we analysed the effect of loss of EBV from Eli-BL clones on growth under optimum conditions and survival in low serum (Figure 3.23). In 10% FCS, Eli-BL clones reached a maximum cell density of around $1.8x10^6$ cells/mL. There was no difference in the proliferation of EBV-positive and EBV-loss clones. In 0.1% FCS we found no proliferation in any Eli-BL clones. EBV-positive and EBV-loss cells died at roughly equal rates and no viable cells remained in the culture after 4-5 days.

Data from growth and survival assays indicates that under optimum growth conditions loss of EBV has little or no effect on proliferation of BL cells. However loss of EBV from the early passage cell line, Mutu-BL and possibly from the intermediate passage cell line Awia-BL reduces the ability of cells to survive in low serum.

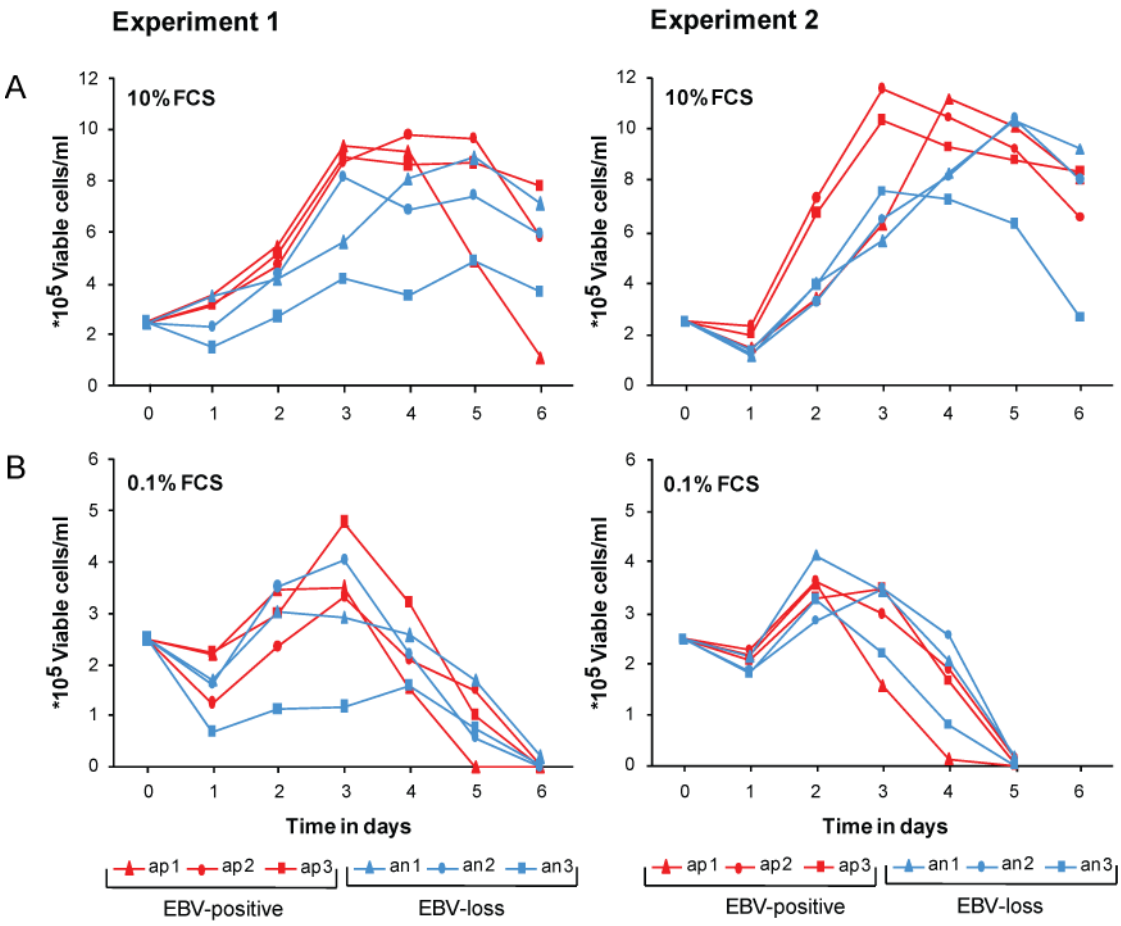


Figure 3.20. Serum dependence of clones of Akata-BL. (A) Growth ability of clones of Akata-BL in 10% FCS. (B) Survival ability of clones of Akata-BL in 0.1% FCS. Values represent average number of viable cells as determined by trypan blue exclusion of multiple aliquots. Number of live cells was recorded every 24 hours for 6 days after the start of the experiment or until no viable cells remained within the culture.

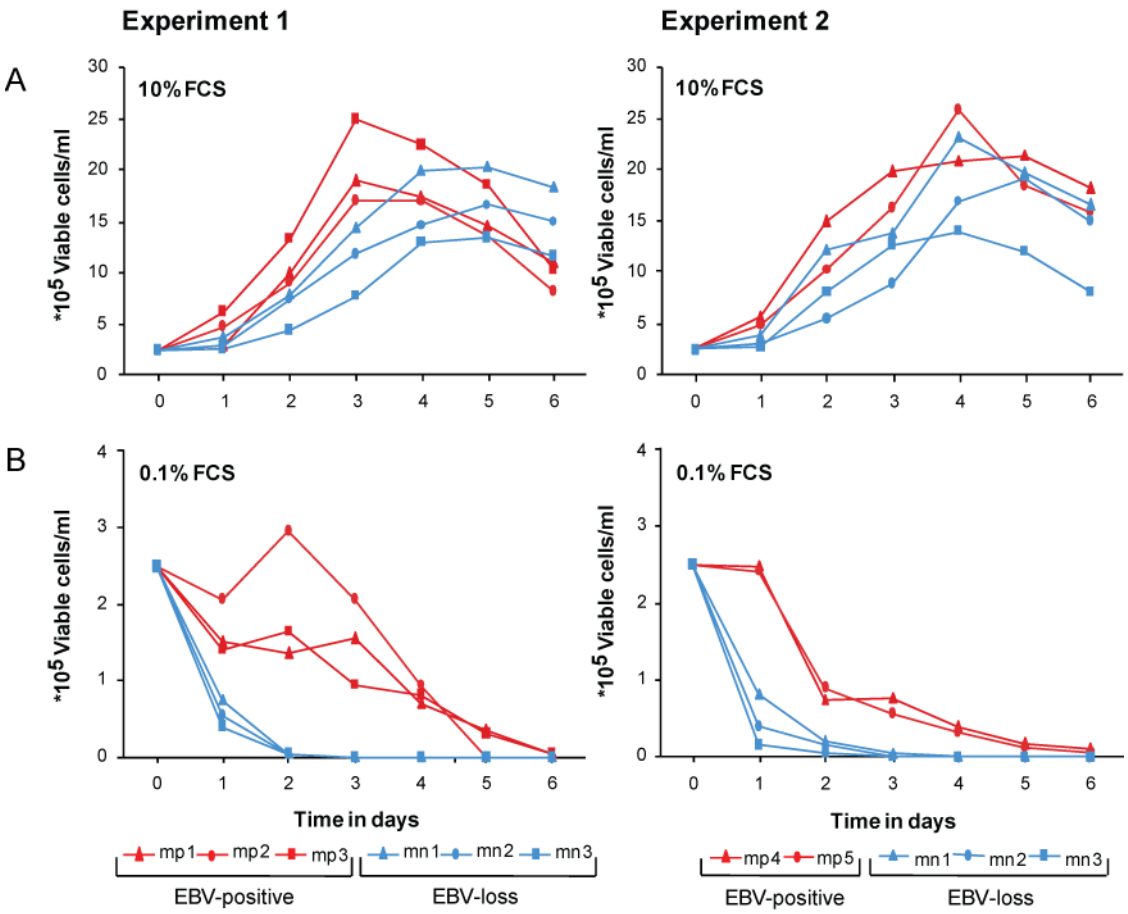


Figure 3.21. Serum dependence of clones of Mutu-BL. (A) Growth ability of clones of Mutu-BL in 10% FCS. (B) Survival ability of clones of Mutu-BL in 0.1% FCS. Values represent average number of viable cells as determined by trypan blue exclusion of multiple aliquots. Number of live cells was recorded every 24 hours for 6 days after the start of the experiment or until no viable cells remained within the culture.

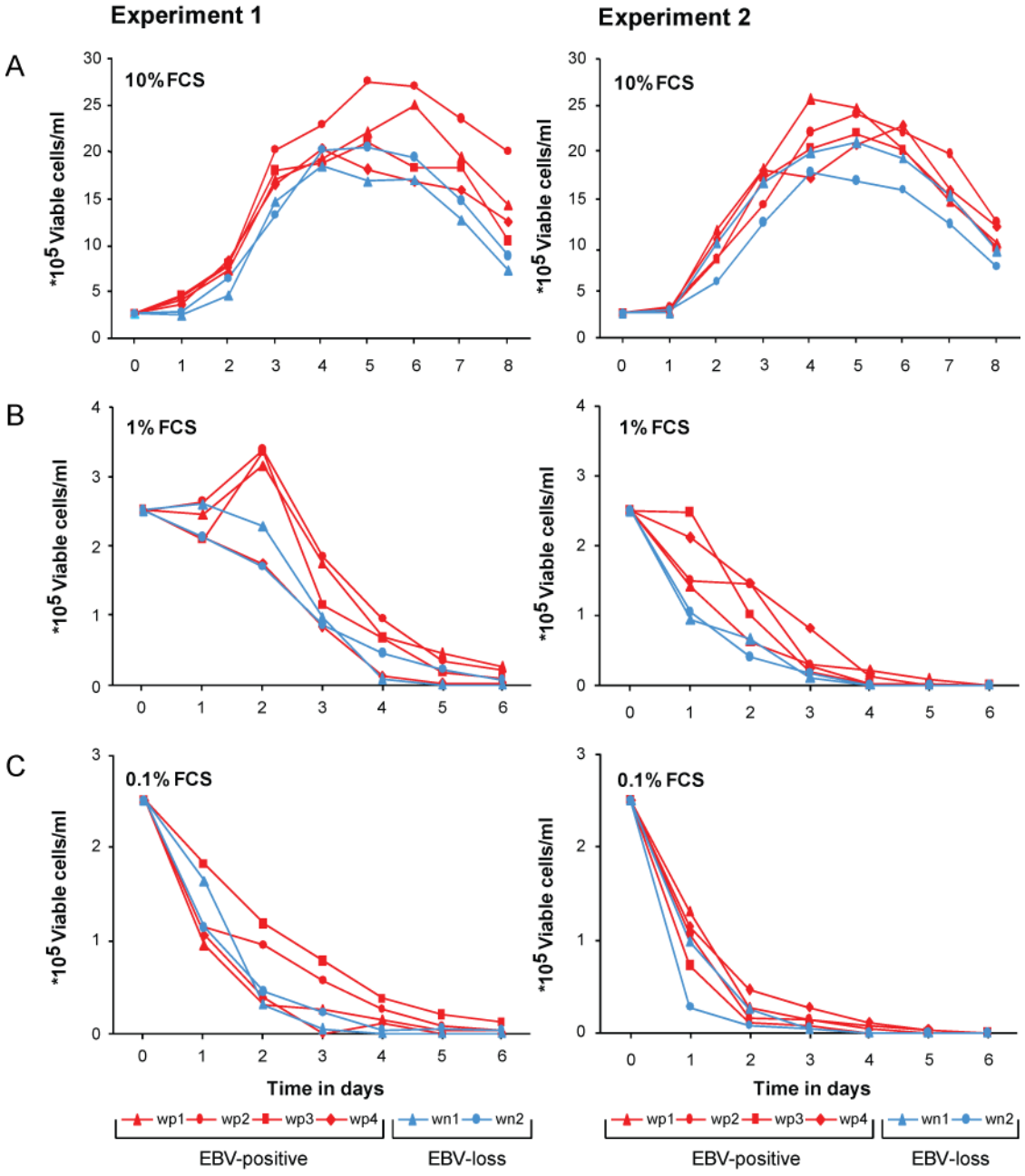


Figure 3.22. Serum dependence of clones of Awia-BL. (A) Growth ability of clones of Awia-BL in 10% FCS. (B) Survival ability of clones of Akata-BL in 1% FCS. (C) Survival ability of clones of Akata-BL in 0.1% FCS. Values represent average number of viable cells as determined by trypan blue exclusion of multiple aliquots. Number of live cells was recorded every 24 hours for 6 days after the start of the experiment or until no viable cells remained within the culture.

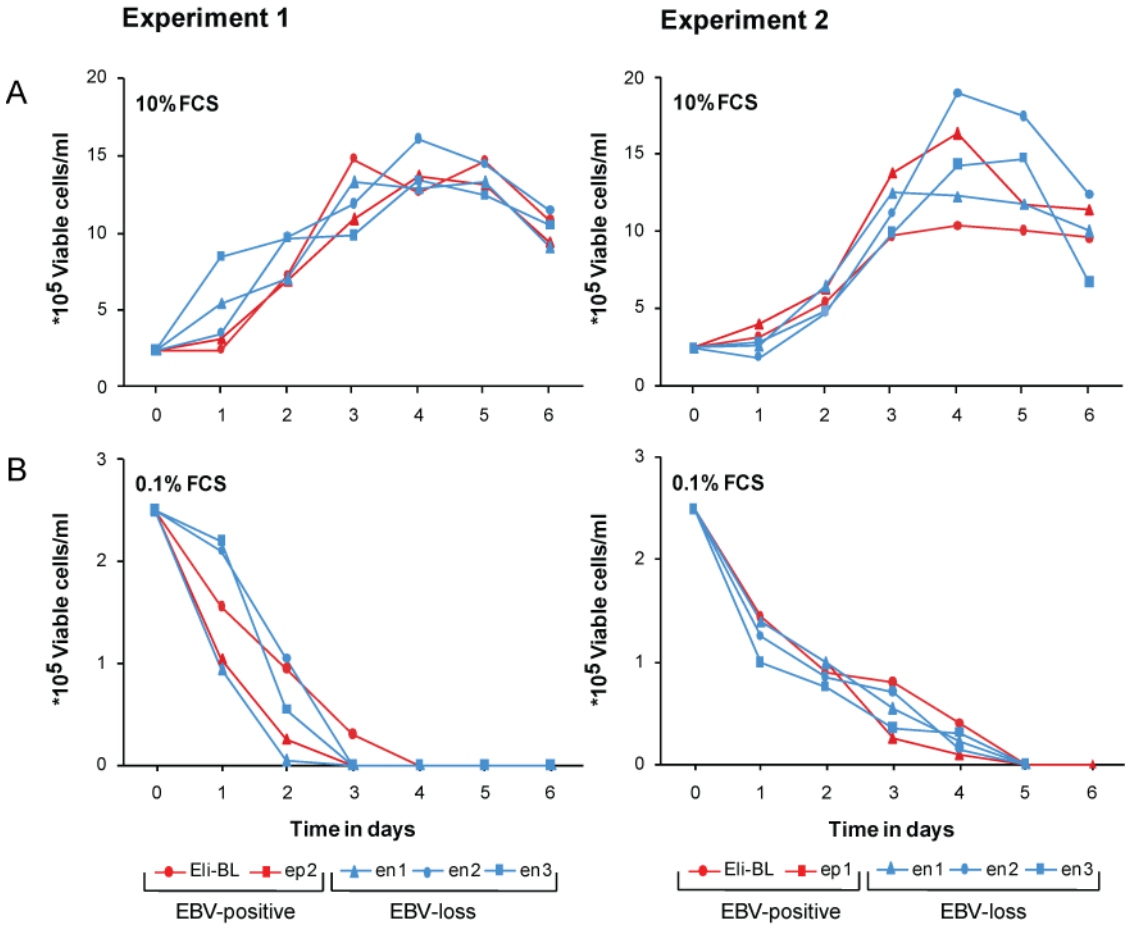


Figure 3.23. Serum dependence of clones of Eli-BL. (A) Growth ability of clones of Eli-BL in 10% FCS. (B) Survival ability of clones of Eli-BL in 0.1% FCS. Values represent average number of viable cells as determined by trypan blue exclusion of multiple aliquots. Number of live cells was recorded every 24 hours for 6 days after the start of the experiment or until no viable cells remained within the culture.

3.5.1 Analysis of cell death induced by low serum concentrations

Following on from the above experiments, we determined whether cells dying as a result of low serum concentrations entered into apoptosis or necrosis. Clones of Akata-BL were resuspended in medium containing 10% FCS, 1% FCS or 0.1% FCS and cultivated in 96 well plates for 72 hours. Cells were then stained with Syto 16 and propidium iodide and analysed by flow cytometry. Figure 3.24 shows induction of apoptosis and necrosis after 72 hours in 1% and 0.1% FCS. In contrast to ionomycin treatment, most of the cell death induced by low serum resulted from necrosis rather than apoptosis. We found no difference in induced cell death between EBV-positive and EBV-loss clones at 1% or 0.1% FCS. To ensure that cells were not dying by apoptosis followed by secondary necrosis, we repeated the experiment with 24 and 48 hour time points. The level of induced cell death in these assays was less than at 72 hours, but still predominantly necrotic indicating that, in clones of Akata-BL, EBV may be able to protect cells from apoptosis induced by ionomycin, but not necrosis induced by low serum concentrations.

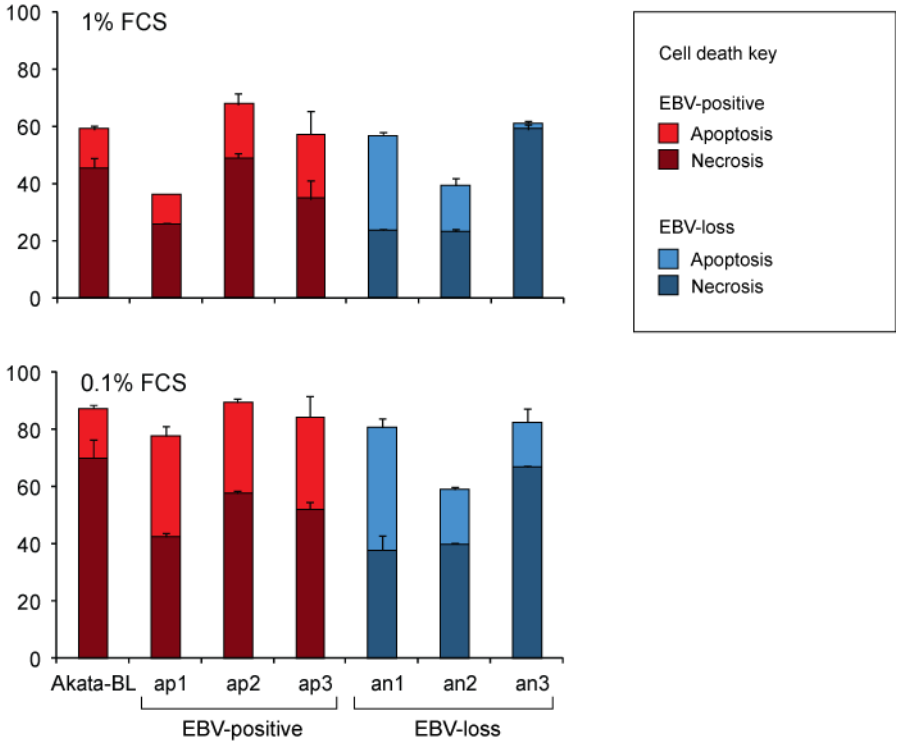


Figure 3.24. Percentage induced cell death induced by low serum in clones of Akata-BL at 72 hours, as determined by Syto 16 and propidium iodide staining (Figure 3.14). The lighter coloured section of each bar represents induced apoptosis and the darker section is induced necrosis. Error bars represent standard deviation in cell death across 3 independent wells.

3.6 Colony forming ability of BL clones in soft agar

We next examined the ability of EBV-positive and EBV-loss clones of Akata-BL, Mutu-BL, Awia-BL and Eli-BL to form colonies in soft agar. As described in 2.5.4, cells were suspended at low densities in 0.33% low melting point (LoMP) agarose and then cooled on 1% LoMP agarose base layers to fix cells within the agarose matrix. Cells were then cultured for 4 weeks to allow the formation of visible colonies. This ability to survive without the support of cell to cell contact provides a measure of tumourigenicity in BL clones (Shimizu et al., 1994). In preliminary experiments, the number of cells required for the formation of colonies in soft agar was determined using a single EBV-positive and EBV-loss clone from each cell line (data not shown). There was a large variation in the number of cells required for colony formation in LoMP agarose between different cell lines; clones of Akata-BL and Mutu-BL required $2.5x10^5$ cells/mL to form colonies, Awia-BL clones required $1x10^5$ cells/mL and Eli-BL clones required only $2.5x10^4$ cells/mL. Once the optimal density for colony formation for each cell line was established, cells from all BL clones were plated out in triplicate wells and cultured for 4 weeks. The number of colonies per well was then recorded and representative fields photographed. At least 2 independent assays were carried out on each of the 4 cell lines investigated.

Figure 3.25 shows anchorage dependence in clones of Akata-BL from 2 independent experiments. All three EBV-positive clones were able to form colonies in soft agar. 2 out of 3 EBV-loss clones produced few or no colonies, but a third EBV-loss clone (an3) generated a similar or greater number of colonies to the EBV-positive clones. In contrast, we found that loss of EBV or a drift to Latency III in Mutu-BL consistently caused a reduction in colony forming ability (Figure 3.26). We found that EBV-loss clones of Awia-BL were unable to grow in soft agar (Figure 3.27). However only 2 out of the 4 EBV-positive clones investigated were able to form colonies. Finally colony forming ability of clones of Eli-BL can be seen in Figure 3.28. We found that colony forming ability varied between clones, but there was no relation between growth in LoMP agarose and EBV status.

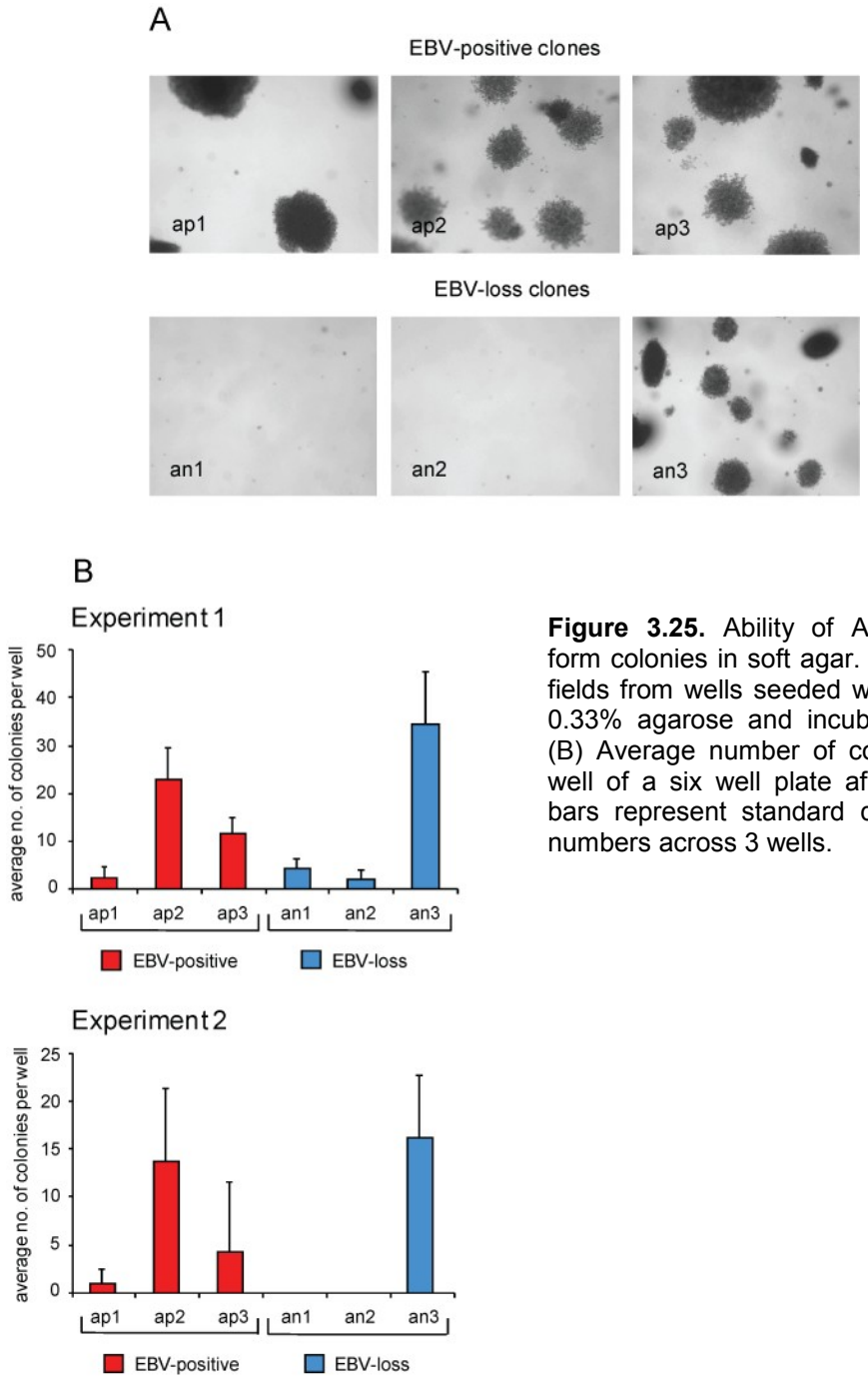
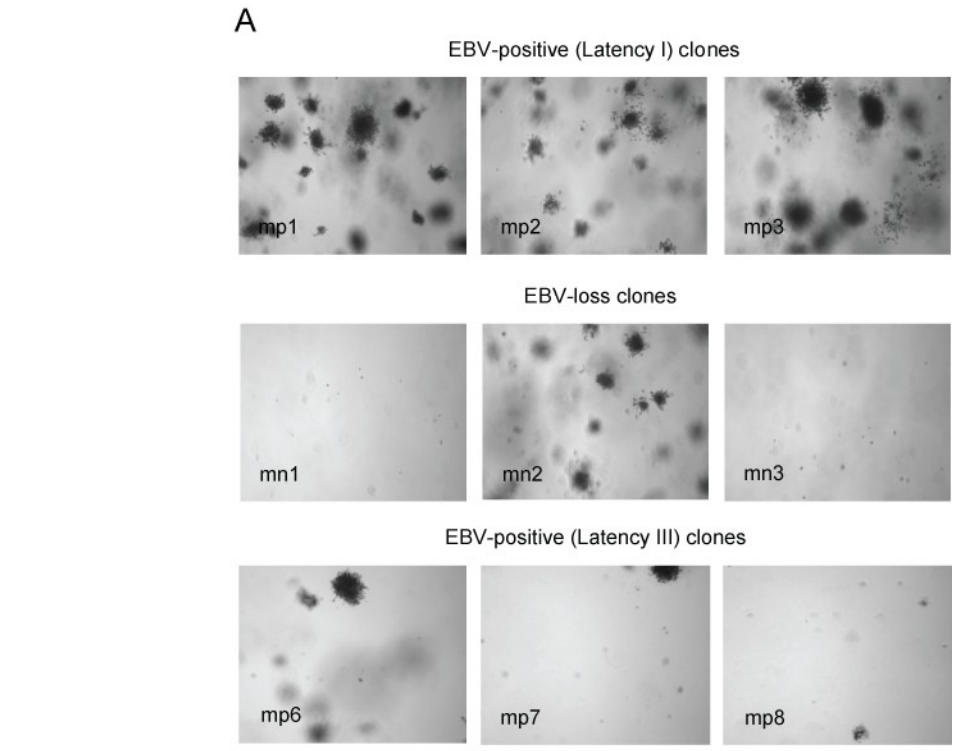


Figure 3.25. Ability of Akata-BL clones to form colonies in soft agar. (A) Representative fields from wells seeded with $2.5x10^5$ cells in 0.33% agarose and incubated for 4 weeks. (B) Average number of colonies formed per well of a six well plate after 4 weeks. Error bars represent standard deviation in colony numbers across 3 wells.



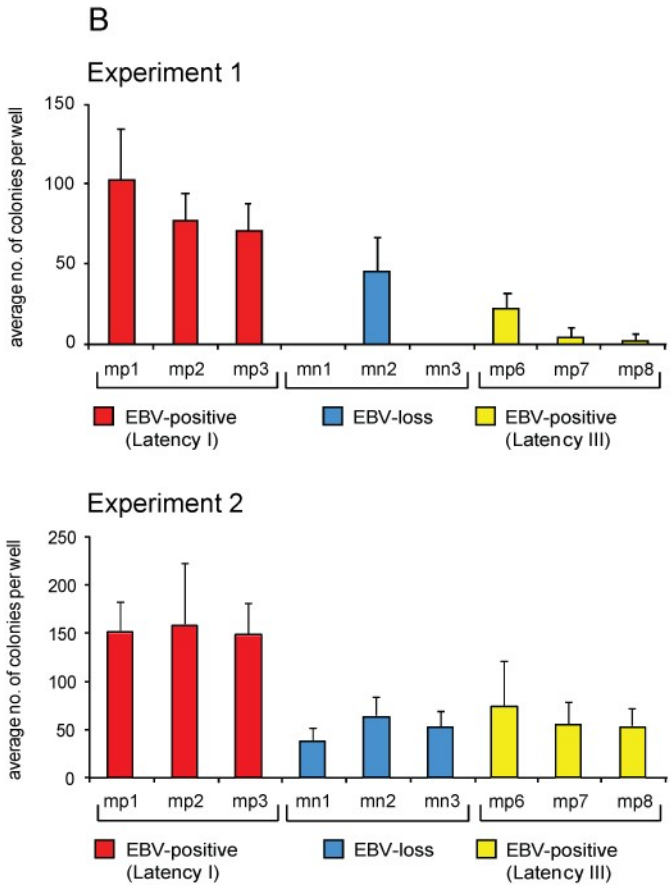


Figure 3.26. Ability of Mutu-BL clones to form colonies in soft agar. (A) Representative fields from wells seeded with $2.5x10^5$ cells in 0.33% agarose and incubated for 4 weeks. (B) Average number of colonies formed per well of a six well plate after 4 weeks. Error bars represent standard deviation in colony numbers across 3 wells.

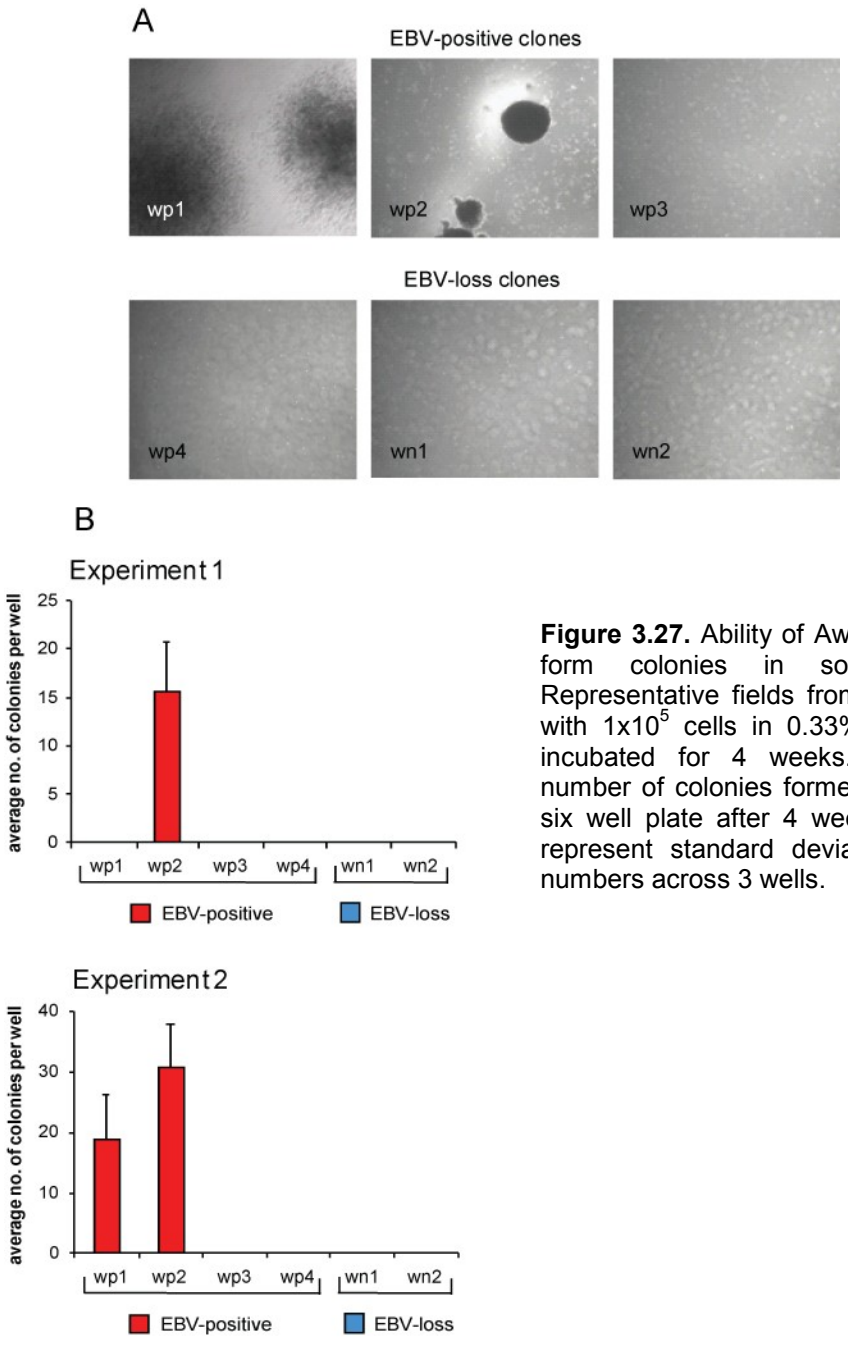


Figure 3.27. Ability of Awia-BL clones to form colonies in soft agar. (A) Representative fields from wells seeded with $1x10^5$ cells in 0.33% agarose and incubated for 4 weeks. (B) Average number of colonies formed per well of a six well plate after 4 weeks. Error bars represent standard deviation in colony numbers across 3 wells.

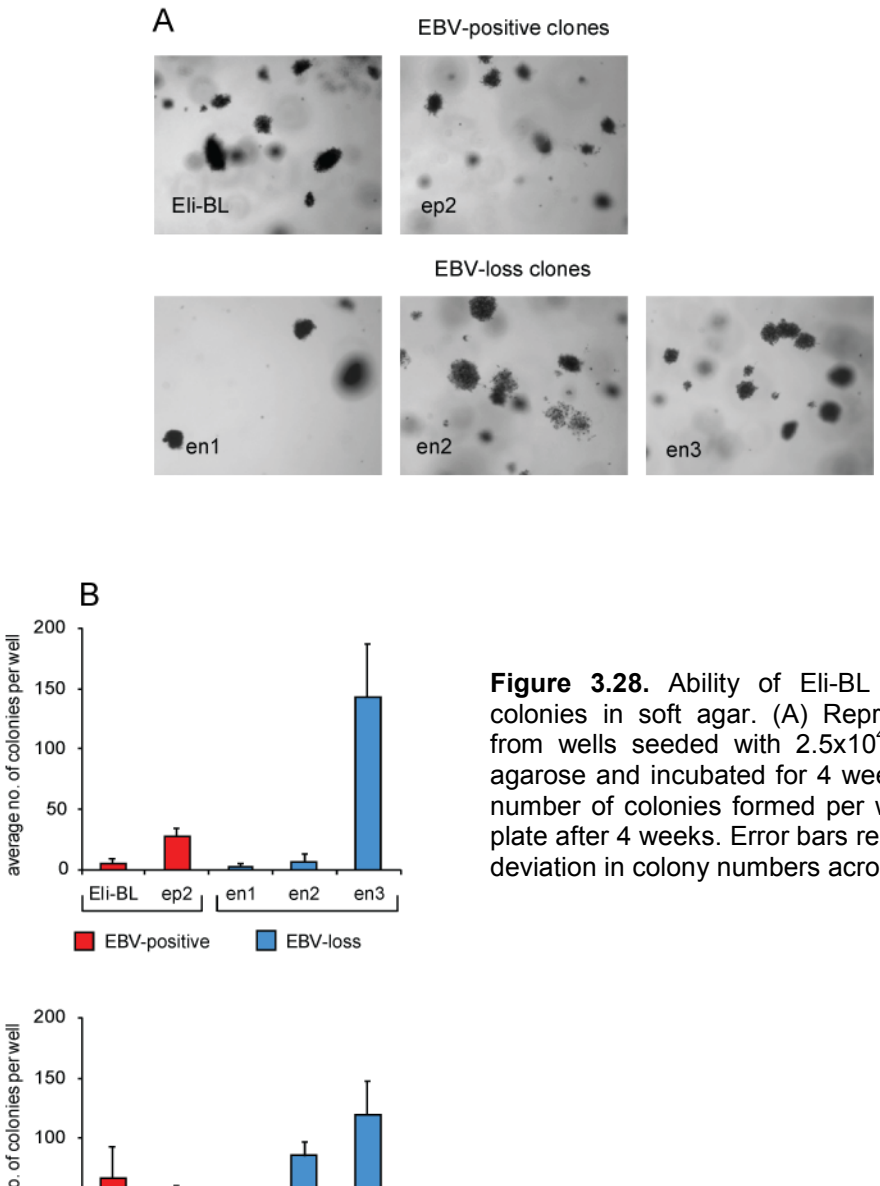


Figure 3.28. Ability of Eli-BL clones to form colonies in soft agar. (A) Representative fields from wells seeded with $2.5x10^4$ cells in 0.33% agarose and incubated for 4 weeks. (B) Average number of colonies formed per well of a six well plate after 4 weeks. Error bars represent standard deviation in colony numbers across 3 wells.

3.7 Western blot analysis of Tcl-1 and Bcl-2 family member proteins

In a final series of experiments, we investigated whether expression of known pro or anti-apoptotic proteins might account for the apoptosis resistance phenotype in EBV-positive BL clones. One protein previously implicated in EBV-mediated resistance to apoptosis in BL cells is the proto-oncogene Tcl-1 (Kiss et al., 2003). Figure 3.29 and Figure 3.30 show western blot analysis of Tcl-1 expression in clones of Akata-BL, Mutu-BL and Eli-BL. In contrast to previous studies, we found strikingly similar Tcl-1 expression between EBV-positive and EBV-loss clones. However we did find the reported down-regulation of Tcl-1 in Latency III clones of Mutu-BL (Kiss et al., 2003).

Another protein linked to apoptosis resistance in BL cells is Bcl-2 (Komano et al., 1998). As described in section 1.11, the Bcl-2 family proteins are known to play a major role in the intrinsic apoptosis pathway; hence we analysed expression of 3 anti-apoptotic Bcl-2 family members (Bcl-2, Bcl-XL and Mcl-1), the pro-apoptotic BH1-3 family members (Bax and Bak) and 3 isoforms of the pro-apoptotic BH3 only protein (BimEL, BimL and BimS). By western blot we found no consistent difference in the expression of Bcl-2 family member proteins between EBV-positive and EBV-loss clones. There was however clonal variation, especially between clones of Akata-BL and Eli-BL. For example a single EBV-loss clone of Akata-BL (an3) expressed a significantly lower level of Bcl-XL compared to all other Akata-BL clones and Bcl-2 appeared to be down-regulated in an EBV-loss clone of Eli-BL (en1).

A useful control again comes from the highly apoptotically resistant, Latency III Mutu-BL clones, which have increased Bcl-2 expression and decreased BimEL expression compared to EBV-loss and EBV-positive Mutu-BL Latency I clones. Although β-actin was an effective loading control for comparing EBV-positive and EBV-loss clones, it too appears to be slightly up-regulated in Latency III clones, an observation which has previously been noted in our lab.

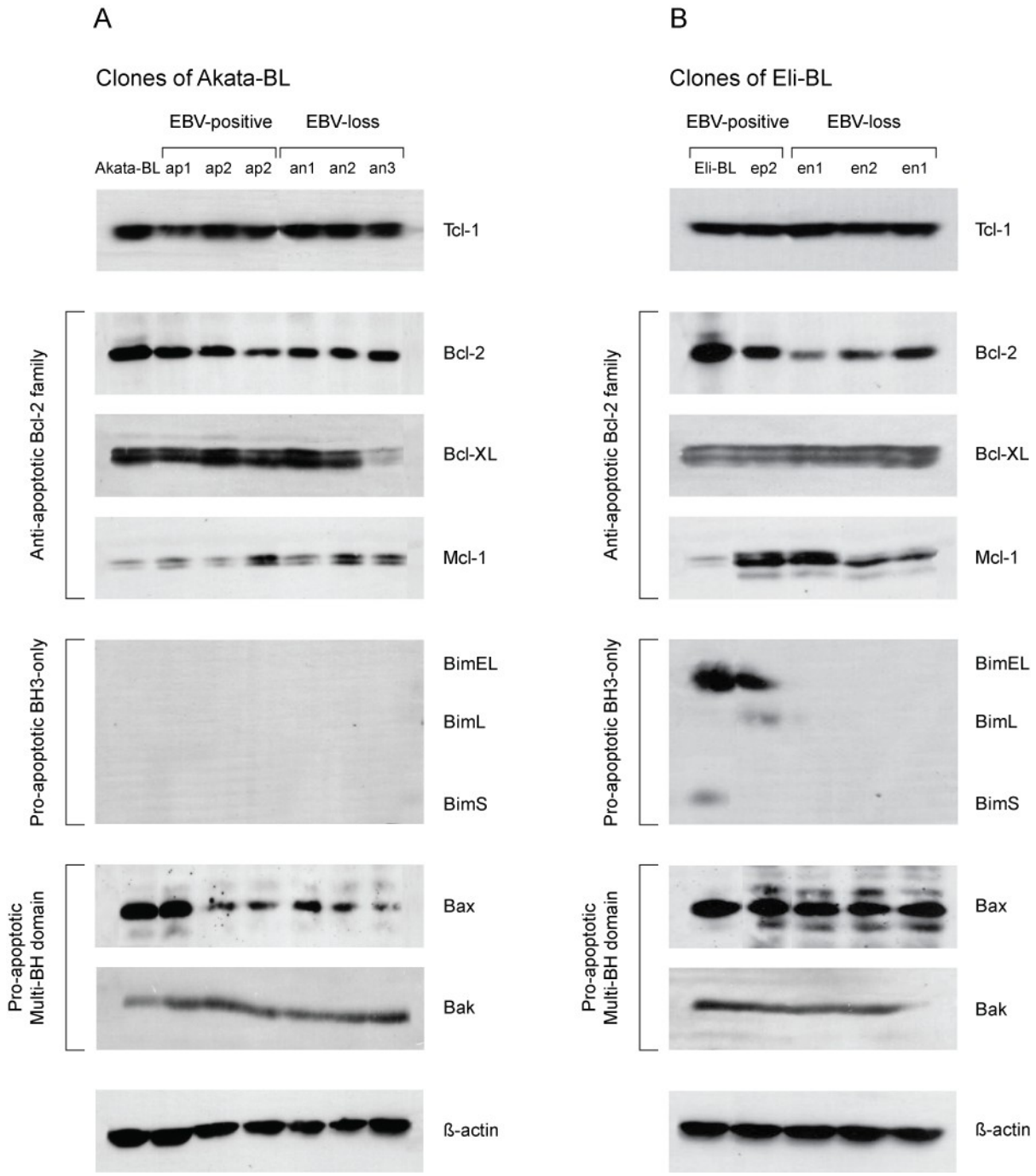


Figure 3.29. Analysis of the expression of the proto-oncogene Tcl-1 and pro and anti-apoptotic members of the Bcl-2 family in EBV-positive and EBV-loss clones of (A) Akata-BL and (B) Eli-BL. Immunoblots were probed with specific antibodies to Tcl-1, the anti apoptotic Bcl-2 proteins (Bcl-2, Bcl-XL and Mcl-1), the 3 isoforms of the pro apoptotic BH3 only protein, Bim and the pro-apoptotic multi-BH domain proteins Bax and Bak. ß-Actin was used as a loading control.

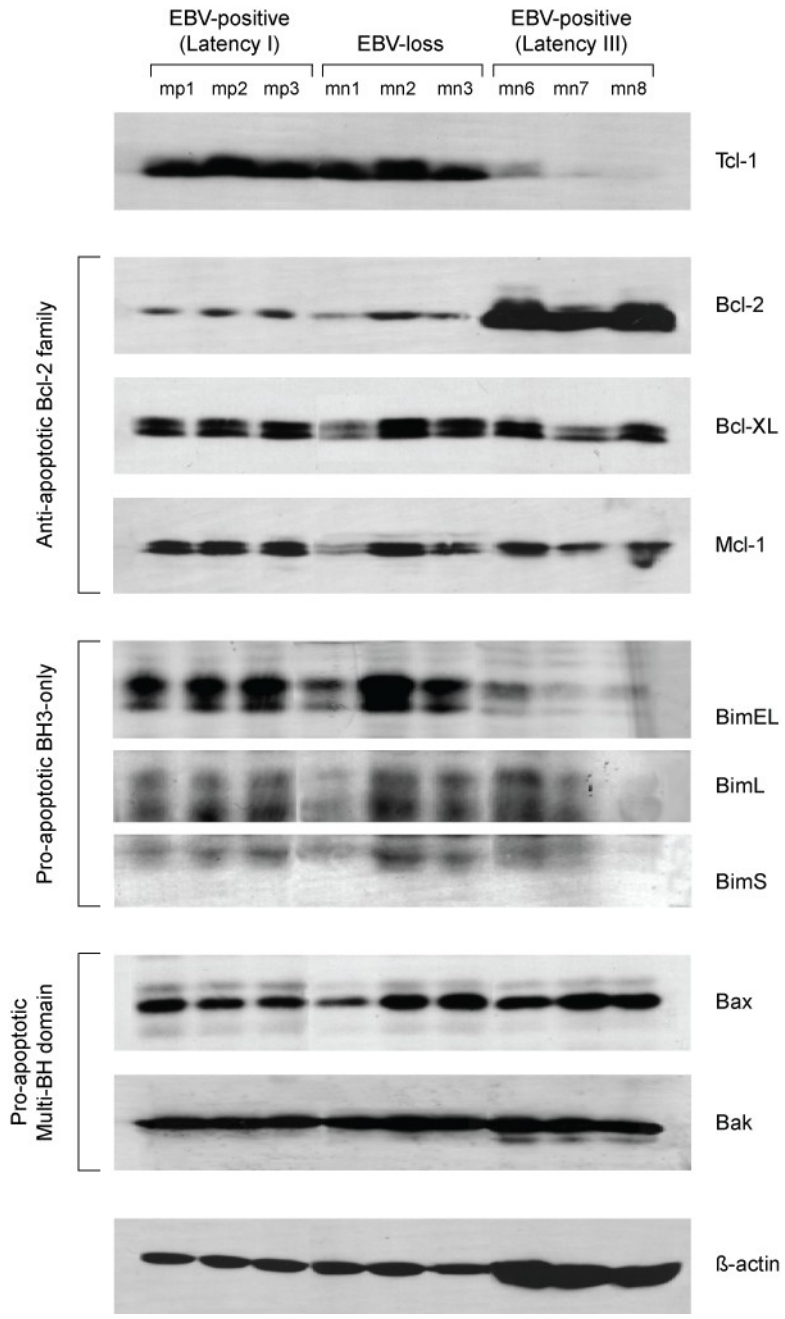


Figure 3.30. Analysis of the expression of the proto-oncogene Tcl-1 and pro and anti-apoptotic members of the Bcl-2 family in EBV-positive Latency I, EBV-loss and EBV-positive Latency III clones of Mutu-BL. Immunoblots were probed with specific antibodies to Tcl-1, the anti apoptotic Bcl-2 proteins (Bcl-2, Bcl-XL and Mcl-1), the 3 isoforms of the pro apoptotic BH3 only protein Bim and the pro-apoptotic multi-BH domain proteins Bax and Bak. ß-Actin was used as a loading control. It should be noted that ß-Actin is up-regulated in Latency III clones.

Discussion I

(a) Single cell cloning of BL lines and frequency of EBV-loss clones

As shown in the previous results section, we initially investigated the frequency with which EBV was lost from a panel of 12 EBV-positive, Latency I BL cell lines from early, mid and late passage. This represents a greater diversity of BL cell lines than previous studies and includes commonly used lines such as Akata-BL plus a range of novel eBL cell lines.

The majority of BL cell lines did not generate EBV-loss clones, indicating that in these cases EBV is absolutely required for continued cell growth. Where EBV-loss clones were observed, they generally made up a very small percentage of the total number of clones screened, indicating that even in those cell lines in which EBV-loss is tolerated, it is still a very rare event. However, we cannot rule out the possibility that many more EBV-loss cells are generated that are unable to survive without the presence of EBV. This possibility could be analysed by performing PCR on individual cells from the parental BL cell lines.

From the three early passage BL cell lines investigated, one eBL line (Mutu-BL) generated a small number of EBV-loss clones. Loss of EBV from Mutu-BL has been described previously (Kitagawa et al., 2000), although the frequency of EBV-loss was not described. In addition clones were themselves generated from a late passage single clone of Mutu-BL and not the early passage parental cell line used in this study. The fact that EBV-loss clones were generated from passage 4 of this cell line suggests that a small number of EBV-loss cells may have been present in the tumour *in vivo*.

Of seven mid passage BL cell lines tested, two cell lines (Ava-BL and Awia-BL) yielded a small number of EBV-loss clones. EBV-loss from Awia-BL has been reported previously (Kelly et al., 2006), while EBV-loss in Ava-BL is a novel observation. Although we observed EBV-loss in Ava-BL, the virus appeared to still be providing essential survival signals as the single EBV-loss clone was not viable without the continued support of a fibroblast feeder layer. It is interesting to note that EBV-positive Ava-BL cells express the Wp restricted form of viral latency, loss of which may be far more detrimental to cell survival than loss of Latency I gene expression (Kelly et al., 2005).

Interestingly, we were able to isolate EBV-loss clones from 3 of the 4 late passage BL cell lines analysed, indicating that the likelihood of EBV-loss rises after a significant period of culture *in vitro*.

This phenomenon was first noted in Akata-BL (Shimizu et al., 1994), which was found to lose EBV only after 2 years in culture. In Shimizu et al., (1994), 16 of the 42 clones investigated were completely EBV-negative, a higher percentage than the 5 EBV-loss clones from 45 clones in this study. We observed a similar correlation of EBV-loss with time spent *in vitro* in Eli-BL and Kem-BL, which were cloned in both mid and late passage. In both cases we found EBV-loss clones only in the late passage cultures. However, as only one EBV-loss clone of Kem-BL was isolated, we cannot exclude the possibility that this was simply a very rare event which we missed on the first round of cloning. In Eli-BL however, the large percentage of EBV-loss clones generated in late passage indicated that over a third of cells may have lost EBV during serial passage *in vitro*.

The increased propensity for cells to lose EBV over time indicates that some cells may acquire mutations which may render EBV no longer essential for cell survival. If, as commonly suggested, EBV promotes growth and survival, then mutations within apoptosis genes such as Bcl-2 family members, oncogenes such as Ras or tumour suppressor genes (TSGs) such as Rb could compensate for the absence of EBV-mediated survival signals. In rare cases, mutations may even lead to an incompatibility with EBV infection and may drive a positive selection for EBV-negative cells. For example, deregulated c-myc expression has been shown to be incompatible with full Latency III transcription (Polack et al., 1996; Schuhmacher et al., 1999; Pajic et al., 2001). In cell lines which have a tendency to drift to Latency III such as Mutu-BL, EBV-negative cells may be selected through the drive to maintain high c-Myc expression; however this is yet to be demonstrated *in vitro*.

Interestingly, EBV-loss clones were not spawned from every BL cell line even in late passage, indicating that there cell survival may not be the only barrier to EBV-loss. We noticed that the likelihood of EBV-loss appeared to correlate with the variation in the viral load of the clones resulting from single cell cloning. This is illustrated in Figure 3.1, where there is considerably more variation in the viral load resulting from single cell cloning of Mutu-BL (which generated a small number of EBV-loss clones) when compared to Dante-BL, from which we were unable to find any instances of EBV-loss. This suggests that during cell division there may be unequal segregation of EBV genomes in those lines where EBV-loss is observed. This hypothesis is supported by fluorescence in situ hybridization (FISH) for the EBV genome which has been previously carried out in our lab (Regina Feederle, unpublished data) on metaphase spreads from a number of BL cell lines. This analysis

revealed frequent unequal segregation of EBV genomes during mitosis in early passage Mutu-BL, Awia-BL and late passage Akata-BL and Eli-BL; the fifth cell line which produced EBV-loss clones (late passage Kem-BL) was not investigated using this technique. Interestingly, the unequal segregation of EBV genomes did not appear to correlate with expression of the genome maintenance protein, EBNA1 (personal communication Gemma Kelly), an observation which has previously been noted in LCLs (Sternas et al., 1990). Not investigated however was expression of the EBV tethering protein EBP2, mutation of which could inhibit stable viral maintenance (Shire et al., 1999).

(b) Analysis of EBV gene expression

For each cell line examined we selected EBV-positive clones with a variety of viral loads plus EBV-loss clones to investigate further. When we repeated the DNA-QPCR used during the initial screening process, we noticed an increase in the viral load of the EBV-positive clones, which is likely to be a consequence of low level activity of the EBV lytic cycle, which was temporarily blocked during single cell cloning by the addition of ACV to the cell medium. In some cell lines we also used a flow cytometric assay to measure the percentage of EBER positive cells within EBV-positive clones. This assay was not truly quantitative, as it was only capable of identifying the presence of EBERs in 85% of Raji-BL cells, which have previously been shown by FISH to carry around 50 EBV genomes within every cell (Anvret et al., 1984; Tierney et al., 2007). However, in EBV-positive clones we found comparable staining to Raji-BL indicating a very high proportion of cells were EBER-positive.

Next we investigated viral gene expression by western blotting and QRT-PCR. EBV-positive clones displayed a typical Latency I pattern of viral gene expression, with low level lytic activity explaining the slight increase in EBV genome load. In addition to EBV-positive Latency I clones we also identified a group of Mutu-BL clones with Latency III gene expression. These clones provided useful controls for phenotypic comparisons of BL clones as they are highly resistant to apoptosis (Gregory et al., 1991).

During the course of the study, we noticed that EBV-positive clones from 2 cell lines, Mutu-BL and Eli-BL, began to lose EBV. Given that these cell lines were initially selected on the basis that the parental cells were able to lose EBV, a slow loss of EBV genomes from EBV-positive clones is perhaps to be expected. However EBV-positive clones from these two cell lines appeared to lose the virus at an accelerated rate so were either replaced or eliminated from the study. This spontaneous loss of EBV

from Mutu-BL has previously been noted (Srinivas et al., 1998) and indicates a degree of genetic instability within this cell line. Reassuringly however, the replacement Mutu-BL clones were found to stably retain the virus during the remainder of the project. To further guard against any possible loss of the genome, BL clones were passaged for no more than 6 months in culture, before being replaced with earlier passage stocks from liquid nitrogen storage.

(c) Effect of EBV on apoptosis resistance

Once paired EBV-positive and EBV-loss clones had been extensively characterised, we investigated the phenotypic effect of EBV-loss. Since loss of EBV has previously been reported to reduce resistance to apoptosis (Komano et al., 1998; Ruf et al., 1999), we investigated apoptosis resistance using treatment with either ionomycin or anti-IgM. Ionomycin was selected because of the well established link between elevated intracellular Ca^{2+} ions and apoptosis (Orrenius et al., 2003; Berridge et al., 1998; McConkey and Orrenius, 1997) and we selected anti-IgM cross-linking as a second inducer of apoptosis, as it represents a possible physiological mechanism of BL cell apoptosis induction *in vivo* (Chen et al., 1999b; Gold and DeFranco, 1987). Loss of EBV consistently sensitised cells to both ionomycin and anti-IgM induced cell death, a phenotype which was reproducible over several independent experiments. The difference in apoptosis resistance was fairly modest in clones of Akata-BL; however it was considerably more striking in the eBL cell lines. This was especially noticeable in the earlier passage cell lines, Mutu-BL and Awia-BL, which may reflect an increased dependence on EBV for apoptosis protection.

In Mutu-BL, Latency I infection was clearly able to protect cells from apoptosis; however it was unable to protect cells to the same degree as the full Latency III infection indicating that protection mediated by latent proteins such as EBNA2 (Lee et al., 2002; Lee et al., 2004), the EBNA3s (Anderton et al., 2008; Leao et al., 2007), LMP1 (Gregory et al., 1991; Henderson et al., 1991; Rowe et al., 1994) and LMP2A (Dawson et al., 2001; Guasparri et al., 2008) supercedes the protection offered by expression of Latency I transcripts.

Contrary to a previous report (Kiss et al., 2003), the increased apoptosis resistance in EBV-positive clones was not consistent with increased protein expression of the Tcl-1 proto-oncogene. In fact we found remarkably similar Tcl-1 protein levels across all EBV-positive (Latency I) and EBV-loss clones,

although we did observe the reported down-regulation of Tcl-1 in Latency III clones of Mutu-BL (Kiss et al., 2003). The difference in Tcl-1 expression between EBV-positive (latency I) and EBV-loss clones may therefore be limited to the fairly small number of clones investigated in this published report.

Another protein reported to be up-regulated by the presence of EBV in Akata-BL clones is Bcl-2 (Komano et al., 1998; Komano and Takada, 2001; Ruf et al., 1999). Again however we found no consistent difference in expression of any of the Bcl-2 family member proteins between EBV-positive (Latency I) and EBV-loss clones. The highly apoptotically resistant Latency III Mutu-BL clones, however, clearly up-regulated the anti-apoptotic Bcl-2 protein and down-regulated the pro-apoptotic Bim protein.

Both ionomycin and anti-IgM ultimately induce apoptosis through release of intracellular calcium ions; hence we decided to investigate whether loss of EBV also sensitised cells to a number of other apoptosis inducing agents. Five alternative cytotoxic drugs were selected because of their well documented induction of apoptosis in BL cells (Anderton et al., 2008; Reeves et al., 2007; Wade and Allday, 2000) and because the mechanism by which they induce apoptosis has been fairly well characterised. Thus, we hoped that patterns of drug sensitivity may provide clues to how EBV may modulate the apoptosis pathway. Interestingly, loss of EBV sensitised cells to cell death induced by only one of these drugs (roscovitine). Ionomycin, anti-IgM and roscovitine have all been shown to be capable of inducing apoptosis via p53-independent mechanisms (Alvi et al., 2005; Chen et al., 1999b; Gil-Parrado et al., 2002), whereas the remaining drugs ultimately induce apoptosis through the p53 pathway (Karpinich et al., 2006; Zhang et al., 2002; Chen et al., 2006; Jiang et al., 2004). This indicates that EBV may be capable of protecting cells from cell death induced through p53-independent mechanisms, but not through p53-dependent pathways. Lack of protection by EBV to inducers of apoptosis which act primarily through the p53 pathway has already been reported in two studies using EBV-positive and EBV-loss clones of Akata-BL (Son et al., 2006b; Son et al., 2006a). In these studies, resistance to both the microtubule inhibiting agent, taxol and the HDAC inhibiting agent, trichostatin A (TSA) induced similar levels of apoptosis in both EBV-positive and EBV-loss Akata-BL clones.

To further investigate this observation we examined protein expression of p53 and MDM2 (Figure 3.19). BL cells frequently show inactivation of p53, either through overexpression of the p53 degrading

protein, MDM2 or via crippling p53 mutations (Lindstrom et al., 2001). Degradation of mutant p53 is very inefficient leading to its accumulation within BL cells. In our BL clones there was little or no difference in expression of p53 or MDM2 between EBV-positive and EBV-loss clones, suggesting that EBV-loss was not a product of differential expression of these genes. Akata-BL cells were p53 null, while the other EBV-loss clone yielding cell lines all expressed the high level p53 expression associated with the mutated gene. In addition p53 sequencing data from our lab and others on the parental BL cell lines indicates that in these lines p53 is mutated and non-functional (Lindstrom and Wiman, 2002; Nagy et al., 2004). It is interesting that drugs which induce p53-mediated apoptosis are able to induce cell death in cells with crippling p53 mutations. It is possible that in these cells apoptosis is induced through the p53 homologues, p63 and p73 (Griesmann et al., 2009; Levrero et al., 2000), hinting at a novel modulation of the apoptosis pathway by EBV in BL cells. Unfortunately these experiments were carried out late in this study and thus could not be investigated further.

(d) Effect of EBV on cell growth and survival

Growth of Akata-BL clones under sub-optimal conditions, such as low serum, is reported to be dependent on the presence of EBV; however all clones were reported to grow equally well under normal growth conditions (Shimizu et al., 1994; Komano et al., 1998). In agreement with these findings, we found little difference in the growth of Akata-BL, Awia-BL or Eli-BL clones in normal growth medium; however, we did observe a modest reduction in the rate of proliferation of Mutu-BL cells after EBV-loss. In low serum concentrations, we found considerable variation between the growth and survival ability of individual Akata-BL clones; however there was no correlation between growth and survival and the presence of EBV. These results correlate closely with the investigation into expression of the bcl-2 apoptosis proteins. There was considerable variation in expression of these proteins, and had we randomly selected clones with differential expression of for example bcl-2, it is more likely we would have observed differences in survival in low serum. This indicates that contrary to previous reports, survival of Akata-BL clones in low serum is not affected by EBV status or that the effect is so small that it is masked by much larger changes in growth and survival caused by clonal variation. In agreement with this, there was no difference in the growth or survival ability of clones of Eli-BL in low serum and only a very small difference in survival of Awia-BL clones, which could be

attributed to the general variability of the experiment. In contrast, loss of EBV from Mutu-BL clones caused a large reduction in survival in low serum, which was observed with both the original set of EBV-positive Mutu-BL clones and their more stable replacements. Mutu-BL cells may therefore rely more heavily on EBV for growth and survival as they have had less time in culture in which to acquire compensatory mutations.

The fact that in most cell lines EBV was able to protect cells from apoptosis inducing agents such as ionomycin, but not from serum withdrawal, indicates a different mechanism of cell death in these two environments. When we investigated this possibility we found that, unlike ionomycin treatment, the majority of cell death from serum withdrawal resulted from necrosis. This supports the observation made during the initial apoptosis assays that in most BL cell lines EBV protects cells from apoptosis, but not from necrosis. However further investigation in a range of cell backgrounds may be required to formally demonstrate this effect.

The next phenotypic property we investigated in the BL clones was their ability to form colonies in soft agar. In previous reports, loss of EBV or a drift to Latency III reduced colony forming ability (Kiss et al., 2003; Shimizu et al., 1994; Komano et al., 1998; Torsteinsdottir et al., 1989). We observed this pattern in Mutu-BL and found a partial association of colony forming ability with EBV infection in clones of Awia-BL. In clones of Akata-BL and Eli-BL, however, we found considerable variation in the ability of clones to grow in soft agar, which did not correlate with EBV status. The results of soft agar assays correlate closely with the investigations into survival in low serum. It appears that in the earlier passage Mutu-BL and possibly Awia-BL clones, EBV may be offering some enhancement of survival signals. However, in the late passage lines (Akata-BL and Eli-BL) the modest effect of EBV may be masked by other cellular changes acquired during cell culture.

It is interesting to note that several clones performed poorly in assays of both survival in low serum and colony forming ability in soft agar. EBV-positive Akata-BL clone ap1, for example, is the first clone to die in low serum and produces fewer colonies in soft agar than the other 2 EBV-positive clones. In addition the EBV-positive Awia-BL clone, wp4, shows reduced growth capacity in low serum and does not form colonies in soft agar, indicating that these two assays measure similar aspects of BL biology. Importantly, the results from the apoptosis and survival assays suggest that, unlike in LCLs and PTLD where EBV is growth transforming, the primary role of EBV in BL cells may be anti-apoptotic.

(e) EBV-loss as a model to study the effect of EBV infection

The use of EBV-loss clones in this study allowed the role of EBV to be evaluated in a range of BL cell lines; however this model is not without its drawbacks. As this system relies on naturally occurring EBV-loss events, we cannot rule out the possibility that EBV is lost due to some cellular compensatory mutation. It is also possible, as observed in other BL cell lines such as Raji-BL (Anvret et al., 1984) or Namalwa-BL (Matsuo et al., 1984), that EBV is partially integrated into the host genome. However, if EBV-loss clones do harbour these genetic changes they obviously do not entirely compensate for EBV as virus loss in five independent cell lines was always associated with increased apoptosis sensitivity.

It is possible that by using alternative methods for generating EBV-loss, such as dominant negative EBNA1 (Nasimuzzaman et al., 2005) or hydroxyurea treatment (Chodosh et al., 1998), we may have been able to induce EBV-loss in a greater number of cell lines. By artificially eliminating EBV from cell lines which do not spontaneously lose EBV, we may have observed greater differences between EBV-positive and EBV-loss clones, such as a more consistent difference in growth and survival ability. However these alternative techniques carry their own limitations; cells expressing dominant negative EBNA1 would require antibiotic selection, which can itself induce changes in cell phenotype, while hydroxyurea treated Mutu-BL cells which lost the virus failed to show any change in cell phenotype (Chodosh et al., 1998). In the future it may be possible to use mouse models such as iMyc$^{E\mu}$ mice (Zhu et al., 2005) to investigate the effect of EBV infection on BL pathogenesis; currently however spontaneous loss from our large collection of eBL samples is still likely to provide the best model of the effect of EBV on BL cells. In summary, despite the drawbacks which all experimental approaches to this question inevitably entail, the results described in this Chapter suggest that EBV has a consistent anti-apoptotic function in BL cells.

4. Results Part II

Analysis of BL clones by gene expression profiling

4.1 Introduction

By comparing the phenotype of EBV-positive and EBV-loss BL clones, we found that loss of EBV sensitised cells to apoptosis induced by ionomycin, anti-IgM and roscovitine. In some cell lines, loss of EBV also appeared to reduce proliferation and survival ability. In this next section of work we wished to examine if these differences in cell phenotype between EBV-positive and EBV-loss clones were the result of EBV-mediated changes in cellular gene expression. To address this, we used Affymetrix microarray analysis to investigate the expression of over 47,000 cellular transcripts in 7 EBV-positive and 7 EBV-loss BL clones. To date, there has been no published comparison of matched EBV-positive and EBV-loss endemic BL clones by gene expression profiling.

4.2 Cell lines used for gene expression profiling

2 EBV-positive and 2 EBV-loss clones from Akata-BL, Awia-BL and Mutu-BL, along with a single EBV-positive and EBV-loss clone of Eli-BL were selected for gene expression profiling (Table 8).

These clones have been extensively characterised in the previous chapter. However, as mentioned in section 3.3.2, EBV-positive Mutu-BL clones mp1, mp2 and mp3 displayed a tendency to spontaneously lose EBV in culture. For this reason, we decided to use the more stable mp4 and mp5 clones previously generated from the same early passage Mutu-BL parental cell line, in the microarray analysis.

Burkitt Lymphoma line	Clone	EBV status
Akata-BL	ap2	+
	ap3	+
	an1	-
	an2	-
Awia-BL	wp1	+
	wp3	+
	wn1	-
	wn2	-
Mutu-BL	mp4	+
	mp5	+
	mn1	-
	mn2	-
Eli-BL	ep2	+
	en2	-

Table 8. Table of BL clones analysed by gene expression profiling and their EBV status.

4.3 Analysis of BL clones by Affymetrix gene expression profiling

The methods used to analyse BL clones by Affymetrix gene expression profiling are described fully in material and methods section 2.7 and a schematic representation of the process is shown in Figure 2.4. Initially, large quantities of highly pure RNA were extracted from BL clones in the exponential growth phase using the TRIzol method. Before processing for microarray analysis, an aliquot of RNA was reverse transcribed to cDNA and used to confirm the pattern of viral gene expression in BL clones. As expected, EBV transcripts were absent from all EBV-loss clones and EBV-positive clones continued to express the restricted Latency I pattern of viral gene expression as shown earlier in section 3.3. A second aliquot of each RNA sample was then reverse transcribed to single stranded cDNA using oligo dT primers. Second strand cDNA was then synthesised and the double-stranded cDNA purified. This double-stranded cDNA was used as a template to generate fluorescently labelled cRNA by *in vitro* transcription, which was purified and fragmented before being hybridised to Affymetrix U133 plus 2.0 microarray chips.

The expression level of each gene was determined by binding of fluorescently labelled cRNA to specific sets of probes present on the surface of the chip. The raw data generated by high resolution scanning of the microarray chips was analysed by The Institute of Cancer Studies bioinformatician, Dr Wenbin Wei. Spurious signals were filtered out and expression values were normalised to account for small differences in RNA input, cRNA quality or for variation in basal chip fluorescence. In an attempt to determine the effect of EBV on cellular gene expression, the normalised expression data was then analysed in several different ways including direct comparison of EBV-positive and EBV-loss clones and comparison to published array data.

4.4 Comparison of gene expression profiles of BL clones to a published molecular Burkitt signature

While this work was in progress, two reports described the gene expression profiling of a large number of BL tumours (Hummel et al., 2006; Dave et al., 2006). The methods and results of both these studies are described in more detail in section 1.13.3.1. Briefly, the aim of both studies was to compare cellular gene expression patterns in sporadic BL tumours and diffuse large B cell lymphomas

(DLBCL), 2 malignancies which have a significant degree of overlap in their morphological and histological appearance, but which require different treatment regimes. Using this approach, the authors hoped to identify novel diagnostic markers to differentiate these 2 tumours. The method used to discriminate between samples of BL and DLBCL in the Hummel et al., (2006) study was to develop a molecular Burkitt Lymphoma (mBL) signature. The mBL signature contains a list of genes which are differentially expressed between samples of BL and DLBCL and an expression value for each gene which most accurately represents the expression found in known BL samples. Comparison of each new test sample to this mBL signature allowed the calculation of an mBL index, which was essentially a probability that the sample was of BL tumour origin.

In collaboration with Stephan Bentink (Institute of Functional Genomics, University of Regensburg), who helped to develop the mBL signature (Hummel et al., 2006), we first compared our data generated from the EBV-positive and EBV-loss clones to the mBL signature. This allowed us to determine if, despite serial passage *in vitro*, EBV-positive and EBV-loss clones retained the expression of genes typically seen in BL tumours and more importantly to determine whether the expression of mBL signature genes was affected by EBV.

Figure 4.1 shows the expression profile of genes which make up the mBL signature in all the mature, aggressive, B cell lymphomas examined in Hummel et al., (2006) compared against the 14 EBV-positive and EBV-loss clones tested in our gene expression profiling studies. Expression of the mBL signature genes in all our BL clones correlated very closely with those samples assigned as mBL tumours, but not with the expression pattern seen in DLBCL samples. Thus it appears that serial passage in culture has not caused BL clones to lose their mBL signature. In addition, correlation to the mBL signature also appeared to be unaffected by whether clones originated from sporadic or endemic BL tumours.

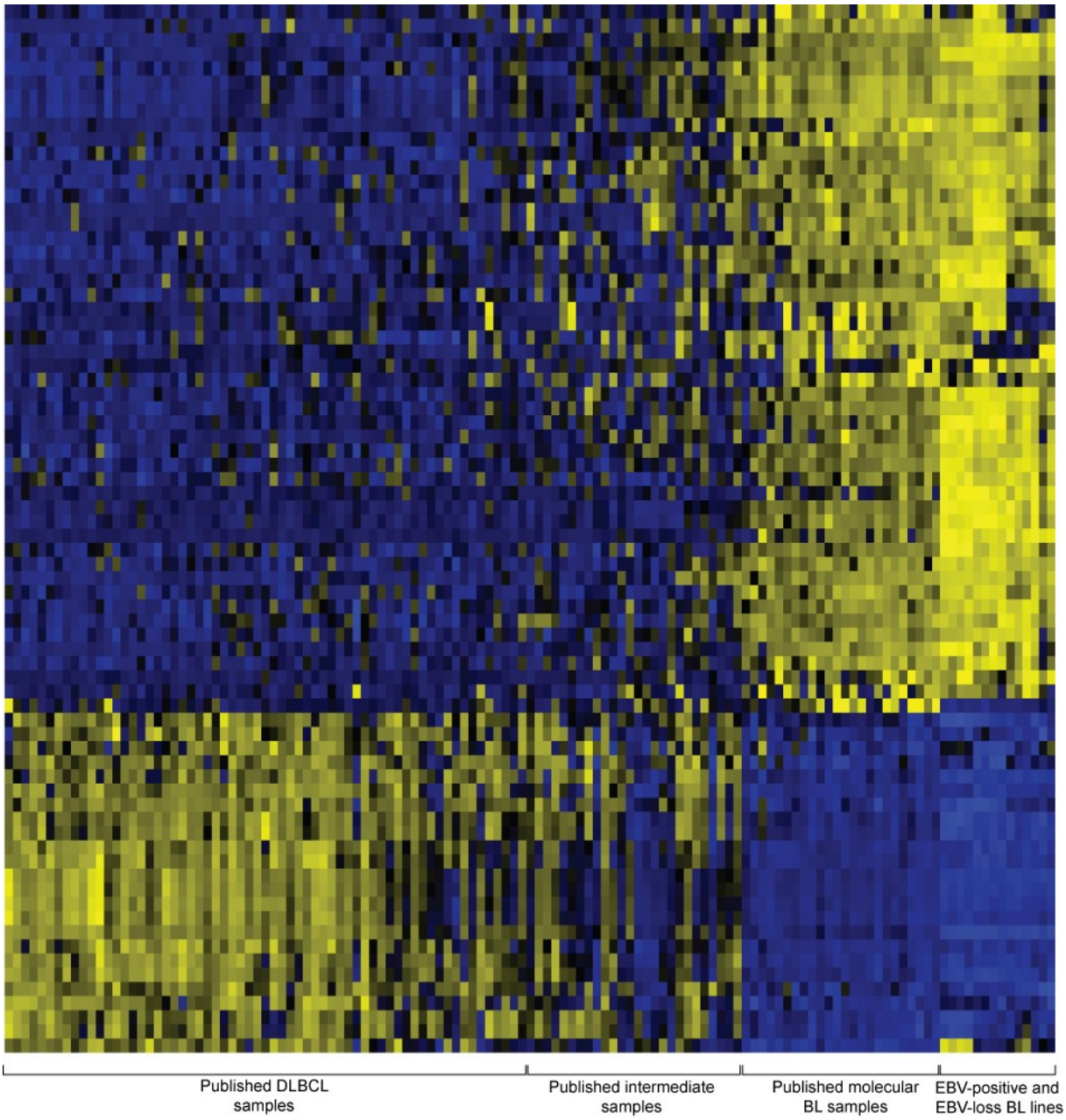


Figure 4.1. Comparison of the gene expression profiles of EBV-positive and EBV-loss clones of Akata-BL, Awia-BL, Mutu-BL and Eli-BL to the large set of aggressive B cell lymphomas described in Hummel et al., (2006). Expression data is shown for the genes identified as molecular Burkitt signature genes and each gene is measured relative to the mean expression across all published samples. Yellow indicates a relative up-regulation compared to this mean and blue indicates a relative down-regulation.

Next we determined if there was any difference in the expression of the mBL signature genes between EBV-positive and EBV-loss clones. Figure 4.2 shows an expanded view of Figure 4.1 with only the gene expression profiles of the experimental EBV-positive and EBV-loss clones shown. This heatmap clearly shows that expression of the vast majority of mBL signature genes is unaffected by the EBV status of the clones. There are 3 exceptions to this pattern, namely UCHL1, TUBB2A and CD40, where there appears to be a small difference between some of the EBV-positive and EBV-loss clones. However, the difference in UCHL1 and CD40 can only be observed in clones of Mutu-BL, while the difference in TUBB2A can only be observed in clones of Mutu-BL and 3 out of the 4 Awia-BL clones. As these changes are not consistent across all clones, it is unlikely that these small isolated differences are biologically significant; hence it appears that the mBL signature genes are not perceptibly regulated by EBV.

Comparison to the published array samples also allowed the mBL index of each BL clone to be calculated (Table 9). All EBV-positive and EBV-loss clones scored well above the 0.95 threshold required to be assigned as mBL samples and in fact several samples correlated so closely to the published mBL samples that they were assigned an mBL index of 1. Interestingly, there was no difference in the mBL index between EBV-positive and EBV-loss clones, indicating that EBV is not the driving force behind the mBL signature of BL cells.

Figure 4.2. Expression of the molecular Burkitt signature genes described in Hummel et al., (2006) in EBV-positive and EBV-loss clones of Akata-BL, Awia-BL, Mutu-BL and Eli-BL. Expression data is shown for the genes identified as molecular Burkitt signature genes and each gene is measured relative to the mean expression across all published samples. Yellow indicates a relative up-regulation compared to this mean and blue indicates a relative down-regulation. The red boxes indicate the Burkitt signature genes where there appears to be a small difference between EBV-positive and EBV-loss clones.

BL cell line	Clone	EBV status	mBL index
Akata-BL	ap2	+	1
	ap3	+	1
	an1	-	1
	an2	-	1
Awia-BL	wp1	+	1
	wp3	+	1
	wn1	-	1
	wn2	-	1
Mutu-BL	mp4	+	0.9999993
	mp5	+	1
	mn1	-	0.9999970
	mn2	-	0.9999999
Eli-BL	ep2	+	0.9999746
	en2	-	0.9999999

Table 9. Table of mBL indices of EBV-positive and EBV-loss clones as determined by comparison to the mBL signature (Hummel et al., 2006).

4.5 Global comparison of gene expression in EBV-positive and EBV-loss clones

After confirming that both EBV-positive and EBV-loss BL clones matched the molecular signature of a BL tumour, we went on to look for genes which may be regulated by EBV. In the first instance, the gene expression of all EBV positive clones was compared to all EBV-loss clones using rank product statistical analysis (section 2.7.10). This statistical analysis, carried out by Dr Wenbin Wei, generated a list of 444 differentially expressed genes. 218 of these genes were up-regulated by EBV and 226 were down-regulated; the names of these genes along with the fold change (FC) and probability of false positivity are listed in Table 10 and Table 11. It is important to note that whilst this list was generated using all 14 BL clones, the genes within it are not necessarily differentially expressed in all 4 tumour backgrounds.

Gene Symbol	Fold change EBV-pos/EBV-loss	Probability of false positivity
CTTN	4.55	0.00
IGLL1	2.61	0.00
PSAT1	2.60	0.00
CTTNBP2NL	2.23	0.00
STXBP6	2.07	0.01
GBA3	2.01	0.00
KIAA0992	1.98	0.01
XBP1	1.98	0.00
CTH	1.95	0.00
MARCKS	1.87	0.02
SHANK2	1.83	0.00
HIST1H3I	1.81	0.00
CKLFSF4	1.80	0.00
FNBP1	1.80	0.00
SPTBN1	1.79	0.00
IQGAP3	1.79	0.01
LTB	1.78	0.00
TNFAIP3	1.74	0.00
CPNE3	1.74	0.07
CACHD1	1.73	0.01
RELB	1.72	0.02
WDR33	1.71	0.01
IL2RG	1.70	0.01
VEGF	1.70	0.00
GNB4	1.70	0.00
ZNF652	1.67	0.00
KLHL9	1.65	0.00
KIAA1622	1.65	0.04
RIMS3	1.64	0.02
STS	1.64	0.00
SLC38A2	1.64	0.01
ST7L	1.63	0.00
SESN2	1.62	0.02
TNFRSF21	1.61	0.04
RPL22L1	1.61	0.00
SPINK2	1.61	0.04
CHST12	1.60	0.07
H2BFS	1.60	0.00
HIST1H2BF	1.58	0.01
SOCS1	1.58	0.01
ZAK	1.57	0.03
CD80	1.56	0.00
WNT5A	1.55	0.01
LY9	1.54	0.04
SLC22A15	1.54	0.07
IL7R	1.53	0.00
BCHE	1.53	0.02
RRBP1	1.53	0.02
SIPA1L2	1.52	0.01
HIST1H3D	1.52	0.05
PIM1	1.50	0.01
CXADR	1.50	0.02
SFRS1	1.50	0.01
LOC92482	1.50	0.05
NCALD	1.50	0.02
BCL2L1	1.49	0.02
WNT2B	1.49	0.01
DDX6	1.49	0.00
CD69	1.48	0.00
PCBD1	1.48	0.03
INHBE	1.47	0.00
IL21R	1.47	0.03
ENOSF1	1.47	0.01
VPS26B	1.46	0.05
SQRDL	1.46	0.07
MALAT1	1.46	0.01
TMEM49	1.45	0.02

Gene Symbol	Fold change EBV-pos/EBV-loss	Probability of false positivity
MGC4504	1.45	0.03
SEZ6L2	1.45	0.09
MRPS6	1.45	0.01
SOX2OT	1.45	0.06
CEBPB	1.44	0.02
ATF5	1.44	0.07
AUTS2	1.44	0.09
BCL3	1.44	0.03
FLJ32416	1.44	0.08
AZGP1	1.43	0.06
PRG1	1.43	0.05
KLF15	1.42	0.08
HIST1H2BE	1.42	0.01
DSU	1.42	0.00
ITGAL	1.42	0.01
SUB1	1.41	0.01
CDKN1B	1.41	0.07
DERA	1.41	0.06
PDCD4	1.41	0.08
FLJ35767	1.41	0.07
HEMGN	1.41	0.09
SMARCD3	1.41	0.09
GNG2	1.41	0.02
MGC4677	1.41	0.08
HIST1H2BI	1.40	0.05
HIST1H2BH	1.40	0.01
HIST1H2BD	1.40	0.03
IGHM	1.40	0.06
OTUD1	1.40	0.01
MGC33584	1.40	0.01
VMD2L3	1.39	0.02
TRIB3	1.39	0.00
SLC7A11	1.39	0.01
PDGFD	1.38	0.06
HCK	1.38	0.04
DOCK9	1.38	0.01
SPAG4	1.38	0.05
FSTL5	1.37	0.08
KIAA1280	1.37	0.04
TAGAP	1.36	0.06
PREX1	1.36	0.04
FN5	1.36	0.05
AVIL	1.36	0.08
CHST11	1.36	0.06
YPEL1	1.36	0.06
PECI	1.36	0.03
WSB1	1.36	0.08
TPK1	1.35	0.03
MYCPBP	1.35	0.02
RAG1	1.35	0.02
RPL27A	1.34	0.07
RIMS2	1.33	0.07
CD53	1.33	0.05
PCK2	1.33	0.03
ZNF532	1.33	0.06
ABHD3	1.33	0.03
DKFZp434L142	1.32	0.05
LOC387816	1.32	0.08
C3orf1	1.31	0.03
FLJ35348	1.31	0.05
FLJ39502	1.31	0.08
BIRC3	1.30	0.00
SNAPC3	1.30	0.07
HIST1H2BM	1.30	0.02
BPGM	1.30	0.01
SLD5	1.30	0.05
B3GNT1	1.29	0.05

Gene Symbol	Fold change EBV-pos/EBV-loss	Probability of false positivity
ZNF275	1.29	0.04
C17orf27	1.28	0.06
GRCC10	1.27	0.01
C9orf55	1.27	0.04
PARP9	1.27	0.08
KIAA0980	1.27	0.06
LOC90693	1.27	0.07
TFDP2	1.27	0.07
CPEB4	1.27	0.03
ARHGEF6	1.26	0.05
GAB1	1.26	0.00
GADD45A	1.26	0.09
WSB2	1.25	0.03
LOC402671	1.25	0.05
INSR	1.24	0.05
SLC39A9	1.24	0.10
PRO1073	1.24	0.05
MTSS1	1.23	0.08
LOC81691	1.23	0.08
LOC400368	1.23	0.05
MSH5	1.23	0.10
FCRL2	1.23	0.07
STAU2	1.22	0.08
ETV6	1.22	0.02
HIST1H1B	1.22	0.07
C14orf4	1.22	0.02
TCP11L1	1.22	0.06
ASZ1	1.22	0.06
SBF2	1.22	0.01
FAH	1.21	0.09
PDE2A	1.21	0.06
PTK2	1.21	0.01
ZNF337	1.21	0.07
NFKBIZ	1.21	0.01
SGPP1	1.20	0.07
DENND4A	1.20	0.03
ERN1	1.20	0.05
DDX11	1.19	0.02
RHEB	1.19	0.04
MAN2A1	1.19	0.10
NEDD4L	1.19	0.05
LMO4	1.18	0.04
KIAA1912	1.18	0.02
LOC90925	1.18	0.09
GRK5	1.17	0.00
KIAA0100	1.17	0.09
CRSP9	1.17	0.04
KIF3A	1.17	0.03
DNMBP	1.16	0.05
CHD2	1.16	0.04
STCH	1.16	0.03
SSBP1	1.16	0.03
ARG2	1.16	0.01
BRD3	1.15	0.04
FRMD3	1.14	0.10
ADA	1.14	0.06
GTF2IRD1	1.14	0.05
DNAJB4	1.14	0.03
RAG2	1.14	0.04
MACF1	1.13	0.10
HSPD1	1.13	0.08
ST8SIA4	1.12	0.01
ACN9	1.12	0.07
WARS	1.12	0.10
KIAA1799	1.11	0.07
MIRN21	1.09	0.00
TCEAL8	1.09	0.00
CTBS	1.09	0.08
RAD54B	1.09	0.10
BRD1	1.09	0.06
CYP39A1	1.08	0.07
MARCH3	1.07	0.09
HSD17B6	1.07	0.01
PLEK	1.06	0.02
IGHV1-69	1.05	0.08
ATP10D	1.04	0.04
RNF11	1.04	0.03
PIGK	1.04	0.06
TAF4B	1.03	0.07
BCL6	1.03	0.08
RAB39B	1.01	0.04
EFCAB2	1.01	0.07
EID-3	1.01	0.05
LOC149832	1.00	0.08

Table 10. Table of genes identified by rank product statistical analysis as up-regulated by EBV.

Gene Symbol	Fold change EBV-pos/EBV-loss	Probability of false positivity
MAPK10	-1.00	0.07
RASSF6	-1.01	0.08
ALDH6A1	-1.01	0.06
SYNE2	-1.01	0.07
LUC7L2	-1.03	0.07
C6orf114	-1.05	0.05
CNOT2	-1.06	0.07
KIAA1279	-1.07	0.04
NRGN	-1.07	0.07
MALAT1	-1.08	0.04
NOTCH2	-1.08	0.02
LOC201895	-1.08	0.10
BNIP3	-1.09	0.07
PKP4	-1.10	0.06
LOC161527	-1.11	0.09
DKFZp547E087	-1.14	0.05
SDCCAG3	-1.15	0.08
EMP3	-1.16	0.06
FBXL7	-1.23	0.03
ZNF559	-1.25	0.09
PLXNB2	-1.25	0.05
UBE2E3	-1.26	0.06
NALP4	-1.26	0.00
TFDP1	-1.27	0.07
LACTB	-1.27	0.10
TGFBRAP1	-1.27	0.09
RBMS1	-1.27	0.10
PNOC	-1.28	0.07
CLYBL	-1.28	0.03
NUDT3	-1.29	0.08
ANUBL1	-1.30	0.07
PTPN12	-1.31	0.02
RHOQ	-1.32	0.08
C9orf10OS	-1.32	0.08
ALOX5	-1.32	0.09
JUP	-1.32	0.05
RAD50	-1.33	0.07
C2orf24	-1.33	0.06
ACPP	-1.33	0.10
NMU	-1.33	0.09
SPP1	-1.33	0.00
CRIP1	-1.34	0.10
OVOS2	-1.34	0.07
TLR1	-1.34	0.07
MATR3	-1.35	0.03
SRL	-1.36	0.02
ZNF395	-1.36	0.09
SLA	-1.36	0.00
SORD	-1.37	0.07
FOLR2	-1.37	0.01
SGEF	-1.37	0.05
STOM	-1.37	0.04
LOC387904	-1.37	0.07
SESN1	-1.37	0.01
ID2 /// ID2B	-1.37	0.00
GMDS	-1.37	0.06
UPF3A	-1.38	0.06
TUSC3	-1.38	0.08
SH2D1A	-1.39	0.01
GYPC	-1.39	0.09
LOC129607	-1.39	0.03
NINJ2	-1.40	0.05
UCHL3	-1.40	0.06
LOC283508	-1.40	0.05
KBTBD7	-1.41	0.01
AGR2	-1.41	0.07
NUP160	-1.41	0.09

Gene Symbol	Fold change EBV-pos/EBV-loss	Probability of false positivity
TTL	-1.41	0.03
LOC285148	-1.41	0.03
GPR75	-1.42	0.07
MAPK13	-1.42	0.01
HDAC9	-1.42	0.00
SYT17	-1.42	0.01
RPS15A	-1.42	0.02
BTLA	-1.43	0.01
TP53BP2	-1.43	0.04
DDX5	-1.43	0.04
FARP2	-1.44	0.08
PRO2964	-1.44	0.01
PDZK3	-1.44	0.02
TSC22D1	-1.45	0.06
ANKMY2	-1.45	0.09
ATP2A3	-1.45	0.09
C20orf59	-1.46	0.07
FLJ11712	-1.47	0.02
LOC93622	-1.47	0.08
FAM80B	-1.47	0.02
CLN5	-1.47	0.04
DLEU2	-1.47	0.02
KBTBD6	-1.47	0.07
EIF2AK4	-1.48	0.04
APRIN	-1.48	0.06
SAP18	-1.48	0.04
LOC441422	-1.48	0.04
PSME3	-1.48	0.05
CD59	-1.48	0.08
LOC440460	-1.48	0.03
DNAJC12	-1.49	0.02
FLJ20186	-1.49	0.01
DOCK8	-1.49	0.05
IFITM1	-1.49	0.09
ZC3H12D	-1.49	0.05
RNF13	-1.49	0.09
RAB40B	-1.49	0.07
MGLL	-1.49	0.03
MRPL1	-1.49	0.08
SH3BP5	-1.49	0.07
ZNF141	-1.49	0.00
KIAA1155	-1.50	0.04
SYNC1	-1.51	0.03
ZNF124	-1.51	0.09
AGGF1	-1.52	0.08
KIAA0114	-1.52	0.06
MLF1IP	-1.52	0.08
GPR18	-1.52	0.02
ATP2B1	-1.52	0.03
EPLIN	-1.52	0.05
KIAA1212	-1.53	0.08
PTPRS	-1.54	0.07
UCHL1	-1.54	0.07
GLB1	-1.54	0.07
STARD3NL	-1.55	0.08
DPYD	-1.55	0.01
ADRBK2	-1.55	0.01
NLN	-1.55	0.03
SLC30A1	-1.55	0.03
NPAL2	-1.55	0.07
KLRK1	-1.56	0.01
PECAM1	-1.56	0.07
PEG10	-1.56	0.03
ABCA11	-1.57	0.01
NDUFS1	-1.57	0.07
ABCC4	-1.59	0.03
ZNF533	-1.59	0.01

Gene Symbol	Fold change EBV-pos/EBV-loss	Probability of false positivity
RGS2	-1.59	0.04
CDC42EP3	-1.60	0.01
MFSD1	-1.60	0.01
H3F3B	-1.60	0.02
C1orf121	-1.60	0.09
CBLB	-1.61	0.06
MIPEP	-1.63	0.03
COTL1	-1.63	0.01
ZNF117	-1.63	0.02
IMMP1L	-1.63	0.07
C14orf139	-1.64	0.03
TFCP2	-1.64	0.05
TMEM23	-1.64	0.07
CAB39L	-1.64	0.07
ADAM23	-1.65	0.00
HPS3	-1.65	0.02
ZC3HAV1	-1.66	0.04
OCIAD2	-1.66	0.03
CYBB	-1.66	0.05
GLIPR1	-1.67	0.03
LOC54103	-1.67	0.03
ID2	-1.67	0.00
RHOQ	-1.67	0.04
LOC116238	-1.67	0.02
LOC285989	-1.68	0.01
ZBTB20	-1.69	0.01
MAPRE2	-1.70	0.02
FAS	-1.70	0.07
ANXA4	-1.70	0.01
C14orf112	-1.70	0.01
MEST	-1.70	0.02
UGT8	-1.70	0.00
C18orf17	-1.71	0.01
FLJ14624	-1.71	0.04
KIAA0674	-1.71	0.06
CD86	-1.71	0.00
CRTAP	-1.72	0.02
KLRC4	-1.74	0.00
TTMP	-1.75	0.08
AIM2	-1.77	0.09
COBLL1	-1.77	0.00
C18orf50	-1.78	0.01
LOC338758	-1.78	0.07
AP3B1	-1.79	0.04
EAF2	-1.79	0.02
EVL	-1.79	0.00
S100A4	-1.81	0.00
PDE4B	-1.82	0.00
ZNF167	-1.85	0.02
QPCT	-1.86	0.03
DST	-1.86	0.07
KLHL5	-1.87	0.01
LOC374443	-1.88	0.03
BANK1	-1.91	0.00
SLC39A10	-1.92	0.00
FLJ20366	-1.92	0.00
SPAG16	-1.92	0.02
BIN1	-1.92	0.00
TMEM38B	-1.93	0.00
CD180	-1.93	0.00
LOC402110	-1.93	0.00
SLC25A13	-1.95	0.03
PCDH9	-1.96	0.00
CLLU1	-1.96	0.07
PEX1	-1.98	0.06
PDLIM1	-1.99	0.03
RGS13	-1.99	0.00
GABRB2	-1.99	0.07
CASP7	-2.00	0.08
KMO	-2.01	0.01
NDFIP1	-2.01	0.01
MARCH1	-2.04	0.00
LYSMD2	-2.08	0.01
SGCE	-2.10	0.03
MGC17943	-2.16	0.01
GNG10	-2.17	0.02
LOC441075	-2.17	0.02
SLC15A2	-2.17	0.00
SNRPN	-2.22	0.00
ZNF529	-2.24	0.00
LOC441168	-2.27	0.00
ELK3	-2.32	0.01
RAPGEF2	-2.59	0.01
C6orf115	-2.63	0.00
MEF2C	-2.68	0.00
NMI	-2.74	0.00
FLJ30596	-2.83	0.00
VPREB1	-2.91	0.00
DMD	-2.92	0.10
SNRPN	-3.00	0.00
FLJ11273	-3.58	0.00

Table 11. Table of genes identified by rank product statistical analysis as down-regulated by EBV.

4.5.1 Supervised cluster analysis of EBV-positive and EBV-loss clones

To gain an impression of the global gene expression in all 14 BL clones we generated heatmaps which show the expression of each of the 444 differentially expressed genes in all the BL clones. To determine the position of each BL clone within the heatmap, we initially used a supervised clustering approach to place all EBV-positive clones in one group and all EBV-loss clones in another. The aim of this approach was to determine if the presence of EBV affects the expression of large numbers of genes across all the BL backgrounds. Figure 4.3(A) is a schematic representation of a heatmap that would be generated if EBV had an overriding effect on gene expression in all the BL clones. Blocks of genes would be differentially expressed across all EBV-positive and EBV-loss clones. Figure 4.3(B) shows the actual results of supervised clustering in EBV-positive and EBV-loss clones. The actual pattern does not mirror the model shown in Figure 4.3(A). Expression of various genes clearly demarcates the boundaries between clones that originate from different BL tumours and not the boundary between EBV-positive and EBV-loss clones. This indicates that there may be large differences in gene expression between clones from different BL tumour backgrounds and only relatively small differences between EBV-positive and EBV-loss clones from the same background.

4.5.2 Unsupervised cluster analysis of EBV-positive and EBV-loss clones

As an alternative approach to analysing the effect of EBV on global gene expression, we performed an unsupervised clustering analysis, where the position of the 14 BL clones was determined not by their EBV status, but by their expression of the 444 differentially expressed genes. The unsupervised clustering algorithm placed clones with the most similar pattern of gene expression together, while those with different patterns of gene expression were placed further apart. As shown in Figure 4.4, clones from the same tumour background and with the same EBV status always clustered together e.g. the 2 EBV-positive Akata-BL clones clustered together, the 2 EBV-positive Awia-BL clones clustered together and the 2 EBV-positive Mutu-BL clones clustered together. The same pattern was also observed in the EBV-loss clones, indicating that there is fairly low heterogeneity between the clones generated by single cell cloning which are derived from the same parental BL cell line and have the same EBV status.

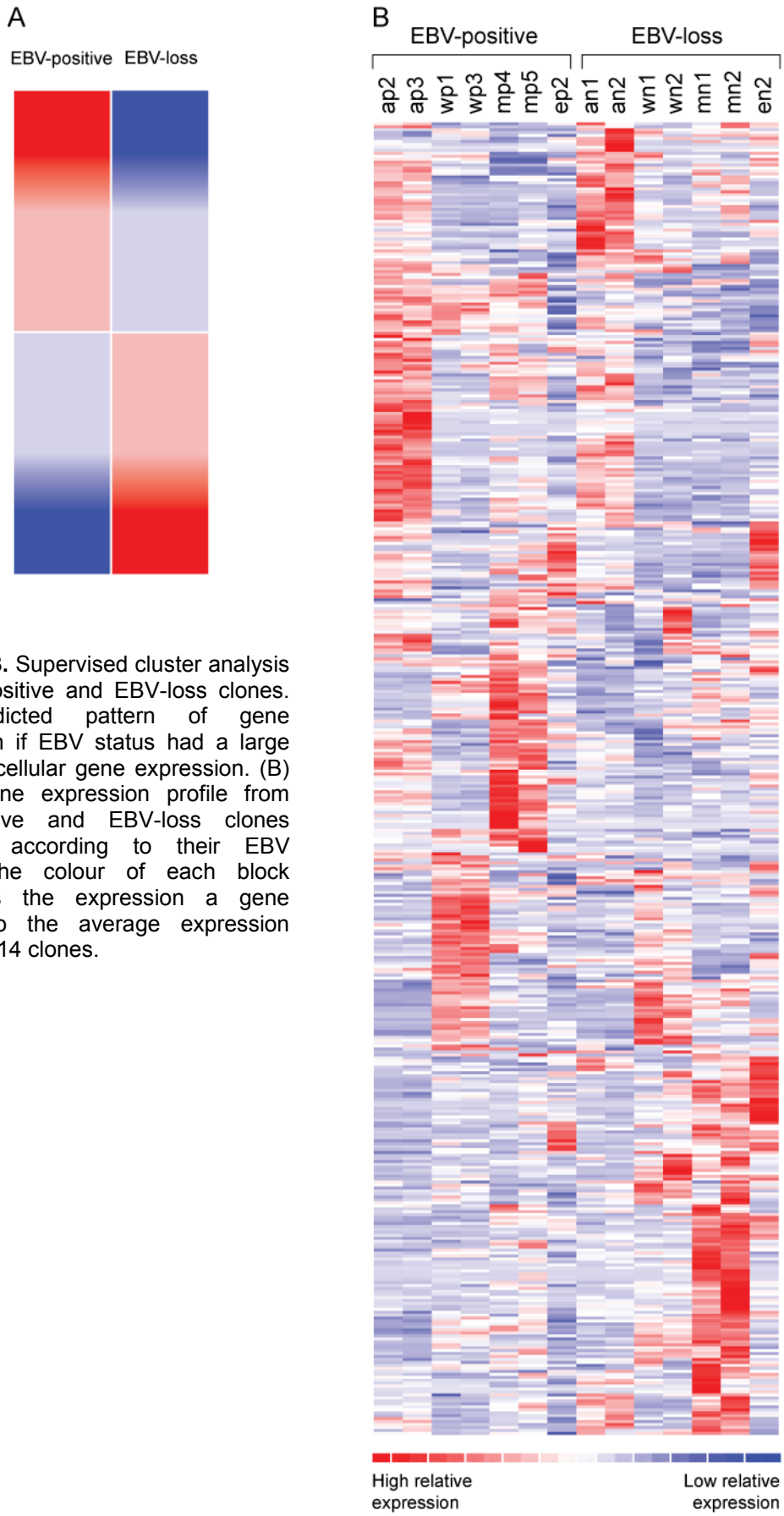


Figure 4.3. Supervised cluster analysis of EBV-positive and EBV-loss clones. (A) Predicted pattern of gene expression if EBV status had a large effect on cellular gene expression. (B) Actual gene expression profile from EBV-positive and EBV-loss clones clustered according to their EBV status. The colour of each block represents the expression a gene relative to the average expression across all 14 clones.

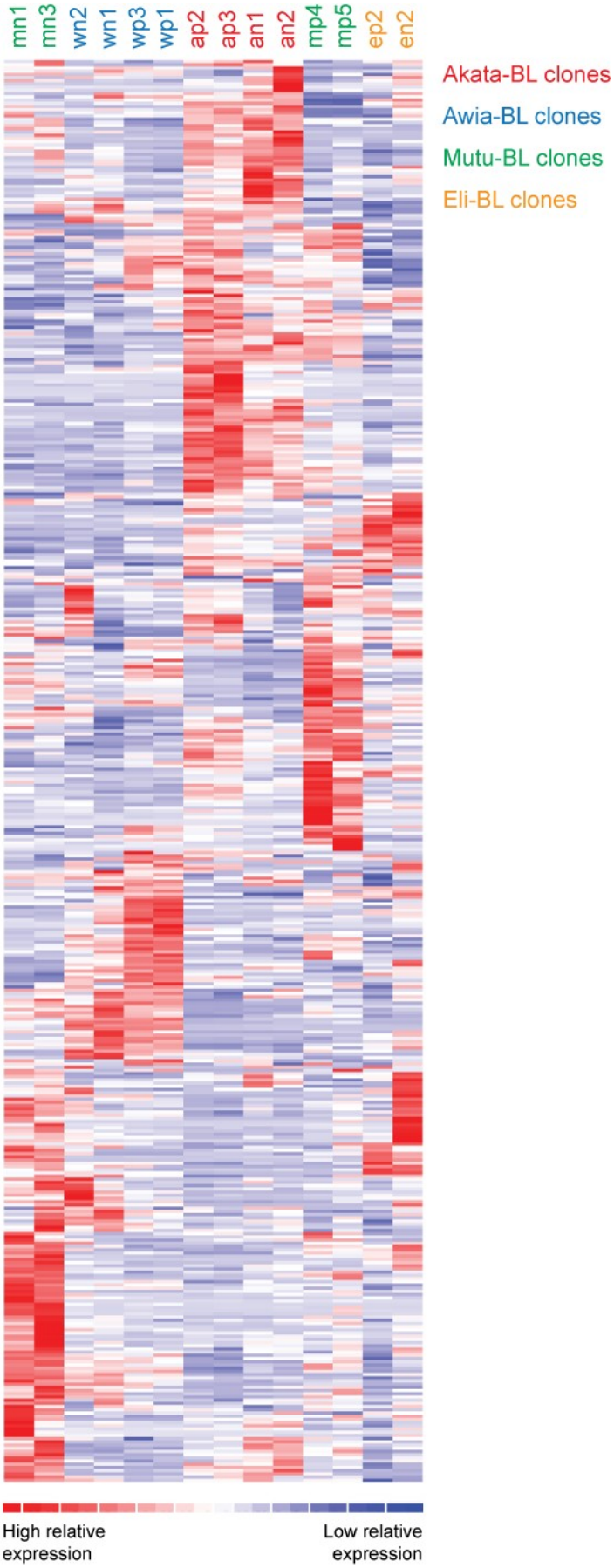


Figure 4.4 Unsupervised clustering of EBV-positive and EBV-loss clones. Clones were allowed to cluster according to their expression of the 444 genes identified as differentially expressed between EBV-positive and EBV-loss clones. The colour of each block represents the expression a gene relative to the average expression across all 14 clones.

If EBV was a major factor in driving cellular gene expression, we might expect all the EBV-positive clones to cluster together in one group and the EBV-loss clones to cluster in another. However, instead of clustering with other EBV-positive clones, we found that EBV-positive clones of Akata-BL clustered with the EBV-loss clones of Akata-BL. Likewise, EBV-positive clones of Awia-BL clustered with EBV-loss clones of Awia-BL and the 2 Eli-BL clones clustered together. The only exception to this pattern was Mutu-BL, where the EBV-positive and EBV-loss clones did not cluster together indicating a greater difference in cellular gene expression.

4.6 Analysis of expression of individual genes using GCOS

While the results of clustering analysis suggested a greater difference in gene expression between clones from different tumour backgrounds than between EBV-positive and EBV-loss clones from the same parental tumour, rank product statistical analysis was still able to find statistically significant differences in gene expression between EBV-positive and EBV-loss clones. To investigate these changes, we used the Affymetrix GeneChip Operating Software (GCOS) to generate bar charts of gene expression for each of the 444 genes identified by rank product statistical analysis.

Representative expression data from 6 genes identified as up-regulated by EBV (TNFAIP3, GNB4, SMARCD3, MARCKS, GBA3 and BCL6) and 6 genes identified as down-regulated by EBV (CD86, FLJ11273, NMI, PCDH9, VPREB1 and UBE2E3) is shown in Figure 4.5 and Figure 4.6. By studying the expression levels of each gene using GCOS, we found that no genes were differentially expressed between all the EBV-positive and EBV-loss clones in all 4 tumour backgrounds. However, there were several genes where differential expression was seen in 2 or 3 of the tumour backgrounds. For example TNFAIP3 was up-regulated in EBV-positive clones of Akata-BL, Mutu-BL and Eli-BL, while GNB4 was up-regulated in EBV-positive clones of Awia-BL and Mutu-BL. CD86 was down-regulated in EBV-positive clones of Akata-BL, Awia-BL and Mutu-BL and FLJ11273 and NMI were down-regulated in EBV-positive clones of Awia-BL, Mutu-BL and Eli-BL, but were not expressed in clones of Akata-BL. However, GCOS analysis also revealed large numbers of genes where there was differential expression in only 1 tumour background e.g. MARCKS, GBA3 and VPREB1 and some genes with differential expression in only a single clone or where the presence of EBV correlated with an up-regulation in one tumour background and a down-regulation in another (BCL6 and UBE2E3).

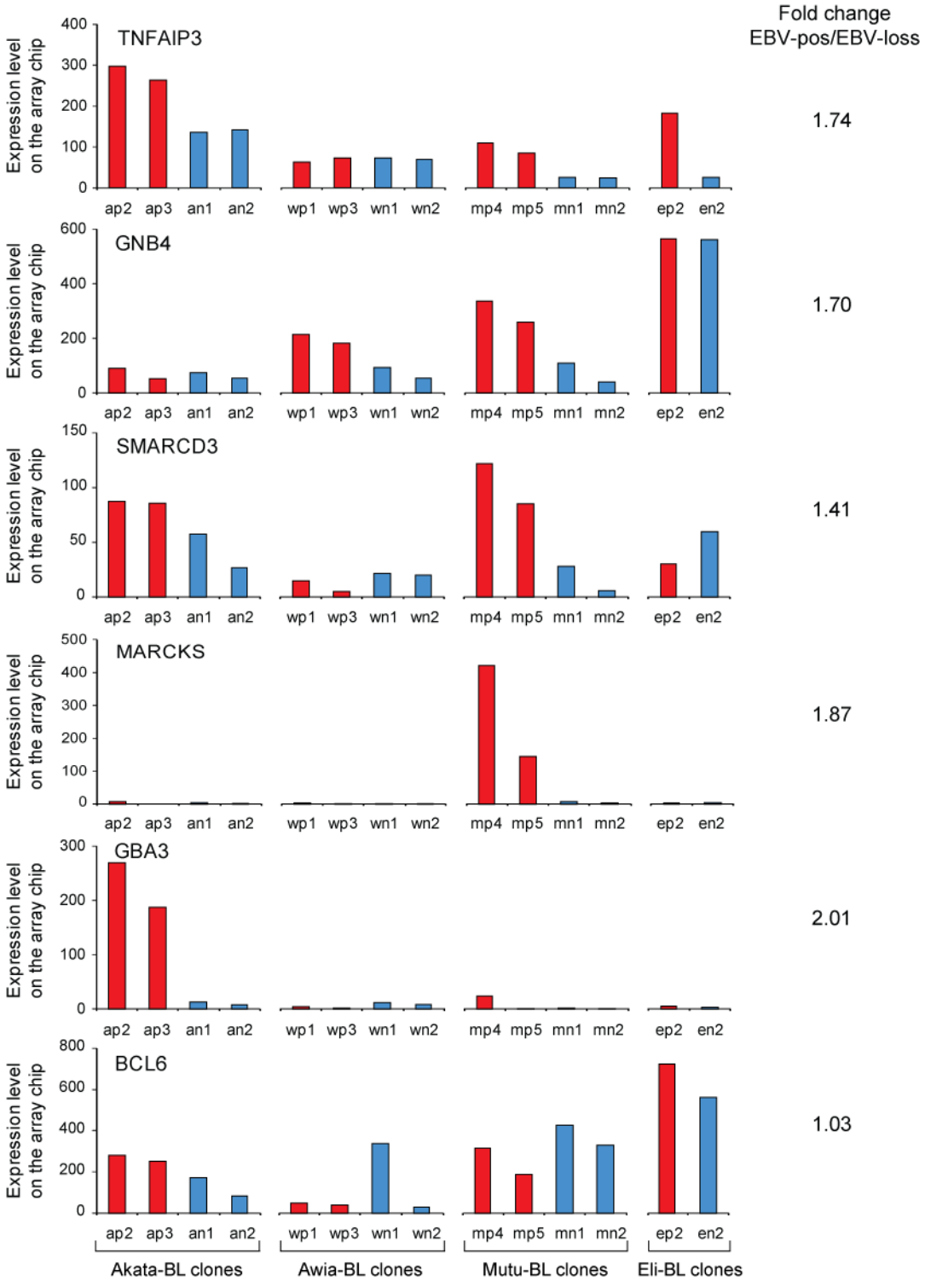


Figure 4.5. Expression data from 6 representative genes identified as up-regulated by EBV by rank product statistical analysis. Expression level was calculated from the original Affymetrix array using the GCOS software.

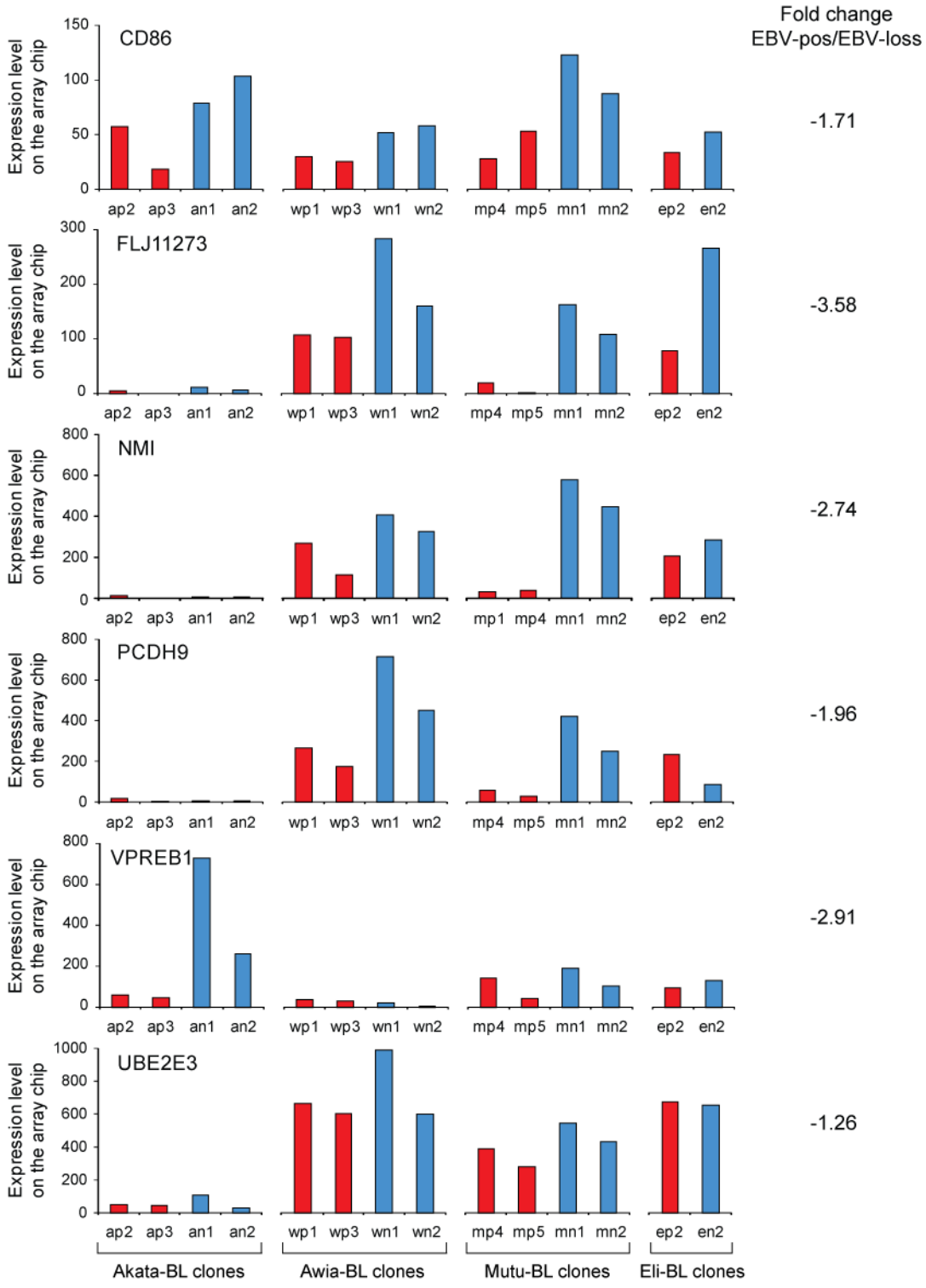


Figure 4.6. Expression data from 6 representative genes identified as down-regulated by EBV by rank product statistical analysis. Expression level was calculated from the original Affymetrix array using the GCOS software.

4.7 Validation of genes differentially expressed between EBV-positive and EBV-loss clones

To validate the gene expression data generated by the GCOS software, we selected a number of the 444 differentially expressed genes and determined their expression at the mRNA and protein level in our panel of 14 BL clones. Genes were selected for further investigation if they were shown by GCOS to be differentially expressed by at least 1.5-fold in at least 2 BL lines and were reported in the literature to play a role in apoptosis. The validation of 3 candidate genes (TNFAIP3, NMI and CD86) is shown below.

4.7.1 TNFAIP3

TNFAIP3 is an anti-apoptotic zinc finger protein, whose expression is rapidly up-regulated in response to receptor binding of pro-apoptotic cytokines from the tumour necrosis factor (TNF) family. TNFAIP3 knockout (KO) mice are highly sensitive to TNF induced inflammatory conditions and cell lines lacking TNFAIP3 are highly susceptible to TNF induced apoptosis (Lee et al., 2000). The anti-apoptotic actions of TNFAIP3 were shown to be a function of its dual activity as a de-ubiquitinase and an ubiquitin ligase. Together these enzymatic functions are believed to block NFκB activation by TNF and thus inhibit TNF induced apoptosis (Wertz et al., 2004).

Figure 4.7(A) shows expression of TNFAIP3 from the Affymetrix microarray chip as determined by GCOS. Figure 4.7(B) and (C) show validation of TNFAIP3 expression by RT-PCR and immunoblotting with a TNFAIP3 specific antibody. As previously shown in Figure 4.5, analysis using GCOS revealed increased TNFAIP3 expression in EBV-positive clones of Akata-BL, Mutu-BL and Eli-BL compared to their respective EBV-loss clones, although no difference was observed in clones of Awia-BL. These differences in TNFAIP3 RNA expression were also observed using the conventional RT-PCR assay described in section 2.8. When TNFAIP3 protein expression was analysed using immunoblotting however, we failed to detect any significant difference between EBV-positive and EBV-loss clones.

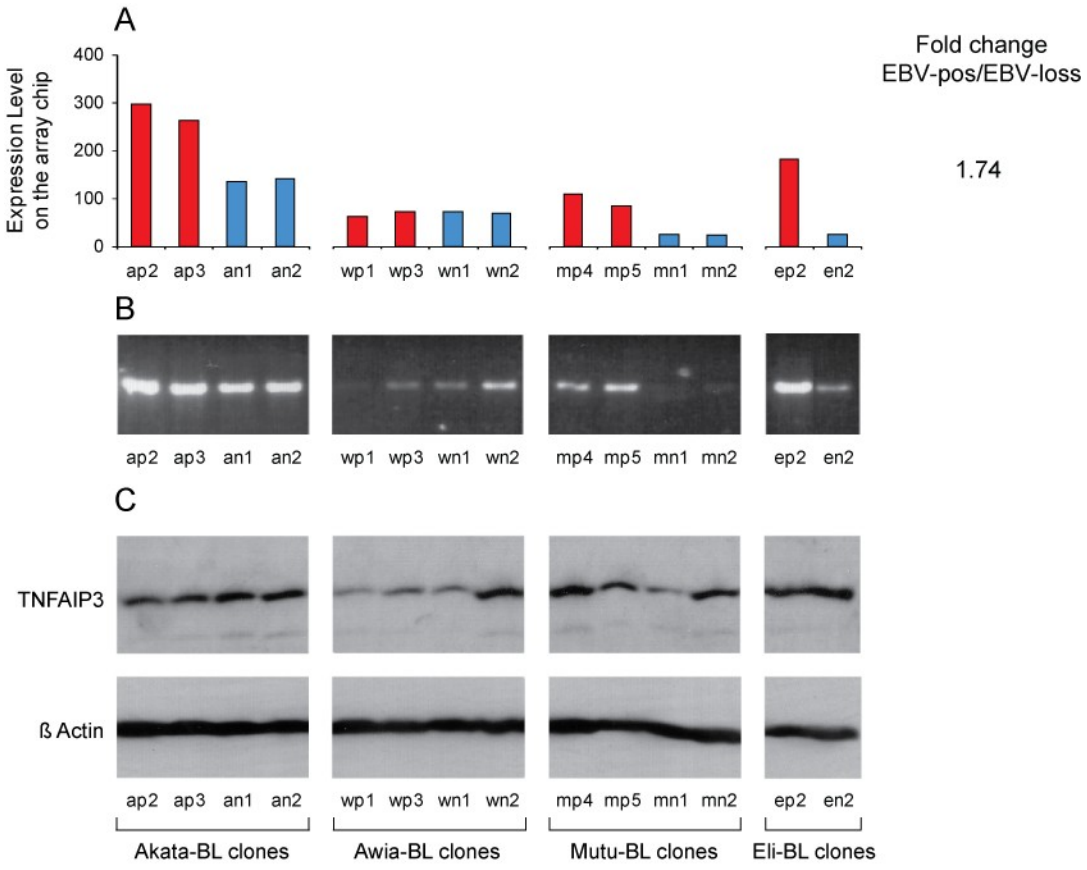


Figure 4.7. TNFAIP3 expression in EBV-positive and EBV-loss BL clones. (A) Expression data generated directly from the Affymetrix chip using GCOS. (B) Conventional RT-PCR for TNFAIP3 expression. (C) Immunoblots probed with an antibody specific for TNFAIP3. ß-Actin was used as a loading control.

4.7.2 NMI

The next gene investigated was N-Myc interactor (NMI), which binds to n-Myc, c-Myc and a number of STAT family proteins (Bannasch et al., 1999). The consequences of NMI binding to Myc proteins have not been fully elucidated; however factors which interact with c-Myc are obviously interesting in the context of a c-Myc driven tumour, such as BL. The STAT family of proteins have wide ranging effects on apoptosis, differentiation and proliferation (Shuai, 2000), but again their regulation by NMI remains unclear.

NMI expression, as determined by GCOS, RT-PCR and immunoblotting with a NMI specific antibody is shown in Figure 4.8(A), (B) and (C) respectively. Using GCOS we found a down-regulation of NMI in EBV-positive clones of Awia-BL and Mutu-BL compared to their paired EBV-loss clones. However, NMI expression appeared to be absent in clones of Akata-BL and there was little or no difference in expression between the clones of Eli-BL. Using RT-PCR we observed the same pattern of gene expression as seen using GCOS so we investigated expression of NMI protein using immunoblotting. There was significant NMI protein expression in EBV-loss clones of Mutu-BL, and little or no expression could be detected in the EBV-positive clones. However NMI appeared to be down-regulated in only one of the 2 EBV-positive clones of Awia-BL. This suggests that while increased resistance to apoptosis in EBV-positive clones of Mutu-BL could be due to a down-regulation of NMI, it is likely that another mechanism is responsible for apoptosis resistance in EBV-positive clones of Akata-BL, Awia-BL and Eli-BL.

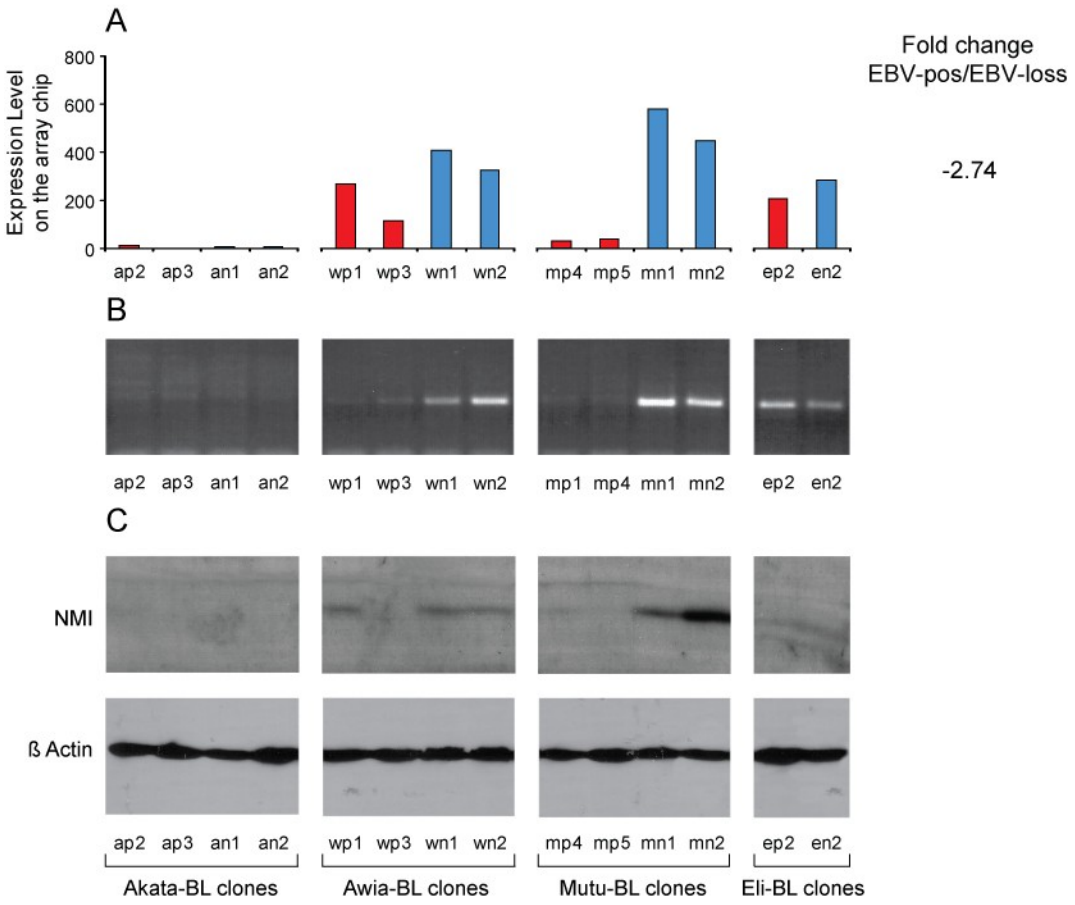


Figure 4.8. NMI expression in EBV-positive and EBV-loss BL clones. (A) Expression data generated directly from the Affymetrix chip using GCOS. (B) Conventional RT-PCR for NMI expression. (C) Immunoblots probed with an antibody specific for NMI. ß-Actin was used as a loading control.

4.7.3 CD86

During the screening of the 444 differentially expressed genes most of the anti-apoptotic genes we identified were up-regulated in EBV-positive clones. However, we also found several anti-apoptotic genes up-regulated in EBV-loss clones, expression of which may help to compensate for the loss of EBV. These included the transmembrane member of the Ig superfamily, CD86, which is expressed on all antigen presenting cells and binds cytotoxic T-lymphocyte-associated protein 4 (CTLA4). In T lymphocytes, binding of CD86 to CTLA4 causes proliferation and increased interleukin-2 production (Jeannin et al., 1997), while in B cells CD86 cross linking increases cellular proliferation and Ig production (Suvas et al., 2002).

CD86 expression, as determined by GCOS, RT-PCR and by flow cytometric analysis following surface staining with a CD86 specific antibody is shown in Figure 4.9(A), (B) and (C to E) respectively. Using GCOS we found a small increase in CD86 expression upon EBV-loss in clones of Akata-BL, Awia-BL and Mutu-BL, but little difference in clones of Eli-BL. In agreement with the GCOS data we found increased CD86 expression in EBV-loss clones of Akata-BL and Awia-BL by RT-PCR, but failed to find any difference in clones of Mutu-BL. When we analysed surface expression of CD86 (Figure 4.9(C)), we found that all BL clones except wn2 were between 90-100% CD86 positive. For this reason we used the mean fluorescence intensity of CD86 staining as a measure of CD86 protein expression. As shown in Figure 4.9(D), there was a small increase in the intensity of CD86 staining in EBV-loss clones of Akata-BL, but this was not observed in EBV-loss clones from the other BL cell lines. Figure 4.9(E) shows the actual flow cytometric data from the clones of Akata-BL; mean CD86 staining intensity is visibly higher in EBV-loss clones, although there is a significant degree of overlap between staining in individual EBV-positive and EBV-loss cells.

Overall, the results of the validation experiments show that RT-PCR data frequently correlated with the data generated using GCOS; however there was often a discrepancy between the expression of a gene at the RNA and protein level.

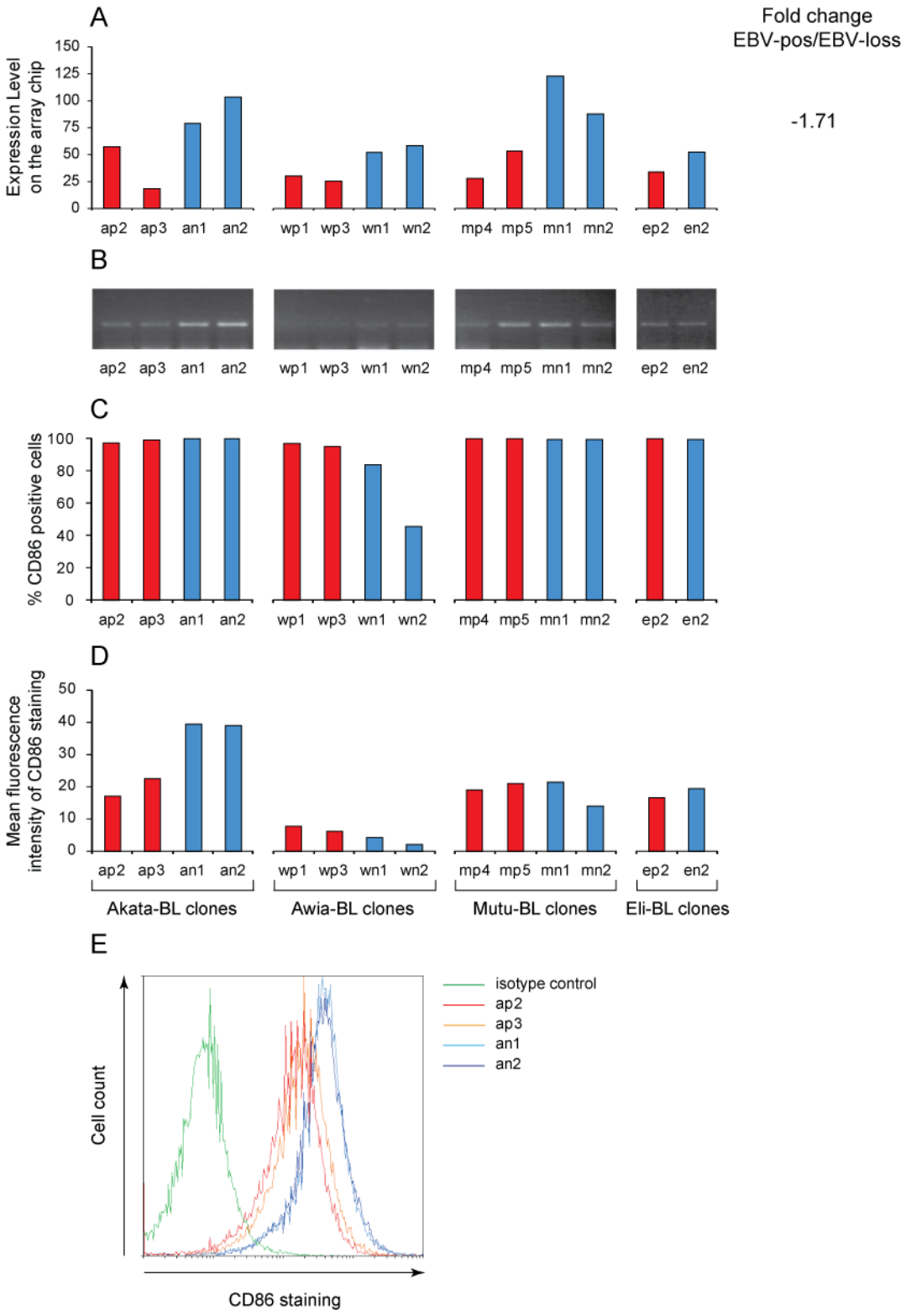


Figure 4.9. CD86 expression in EBV-positive and EBV-loss BL clones. (A) Expression data generated directly from the Affymetrix chip using GCOS. (B) Conventional RT-PCR for CD86 expression. (C to E) Flow cytometric analysis of CD86 expression. (C) Percentage CD86 positivity of cells. (D) Mean fluorescence intensity of CD86 staining. (E) CD86 staining in the EBV-positive and EBV-loss Akata-BL clones compared to isotype staining of the CD86-positive LCL, X50-7.

4.8 Pairwise analysis of gene expression in individual BL cell lines

As we were unable to identify any common genes which were regulated by EBV in all BL tumour lines examined, we next focused on genes which were differentially expressed between EBV-positive and EBV-loss clones in each line in turn. Using this approach it was hoped that we might find clues to common pathways that EBV may regulate, even if the effect was not always moderated through a single gene. The gene expression in the 2 EBV-positive clones of Akata-BL was compared to the 2 EBV-loss clones using rank product statistical analysis and the process was repeated in clones of Awia-BL and Mutu-BL. The Eli-BL clones could not be compared in isolation because there was only a single EBV-positive and EBV-loss clone. Table 12 shows the number of genes that were identified as differentially expressed in clones from the individual tumours. When BL clones from only a single cell line were analysed there was a reduction in the number of samples from 14 to 4. This reduction in statistical power and the loss of the 2 clones of Eli-BL resulted in a reduction in the total number of genes identified as statistically significant from 444 to 293.

When we investigated the BL backgrounds individually we found: 75 genes were differentially expressed between clones of Akata-BL (41 genes up-regulated by EBV and 34 genes down-regulated by EBV), 22 genes were differentially expressed between EBV-positive and EBV-loss clones of Awia-BL (15 genes up-regulated by EBV and 7 genes down-regulated by EBV) and 196 genes were differentially expressed between EBV-positive and EBV-loss clones of Mutu-BL (110 genes up-regulated by EBV and 86 genes down-regulated by EBV). The greater disparity in gene expression between EBV-positive and EBV-loss Mutu-BL clones compared to the other tumour backgrounds was also evident during unsupervised clustering (Figure 4.4) where, unlike clones of Akata-BL, Awia-BL and Eli-BL, EBV-positive and EBV-loss Mutu-BL clones did not cluster together.

	No. of genes up-regulated by EBV	**No. of genes down-regulated by EBV**
Akata-BL	41	34
Awia-BL	15	7
Mutu-BL	110	86

Table 12. The number of genes differentially expressed between EBV-positive and EBV-loss clones from individual BL tumour lines.

We looked next for differentially expressed genes, which were common between 2 or more BL backgrounds, by determining the overlap between the lists of differentially expressed genes. The results are shown as Venn diagrams in Figure 4.10. Remarkably, there are very few common genes and, as expected from the clustering analysis, no genes were identified as differentially expressed across all the tumour backgrounds. In fact only 10 genes from a possible 293 were identified as differentially expressed in 2 out of the 3 different tumour backgrounds. The function of these genes is outlined in Table 13. Of these 10 genes, the majority had functions which were unknown or that were unlikely to contribute to the EBV-positive BL phenotype. ETV6 promotes apoptosis, but it is up-regulated in EBV-positive clones so its expression would not explain apoptosis resistance in EBV-positive clones. ID2, however, promotes apoptosis and is down-regulated in EBV-positive clones; hence we decided to investigate ID2 expression using QRT-PCR and immunoblotting.

Differentially expressed gene	Function
IGLL1	Encodes one of the surrogate light chain subunits of the pre-B cell receptor.
CTTN	Promotes cytoskeleton rearrangement.
CTTNBP2NL	Unknown, homology to the actin binding protein CTTNBP2.
ETV6	Transcription factor involved in haematopoiesis which promotes apoptosis.
ID2	Transcriptional regulator, which inhibits B cell differentiation and promotes apoptosis.
C5orf33	Unknown.
COBLL1	Unknown.
ADAM23	Member of the disintegrin and metalloprotease domain family, which promotes cell adhesion.
CD180	B cell surface receptor which recognises lipopolysaccharide (LPS) on the membrane of Gram-negative bacteria.
EVL	Promotes actin filament rearrangement.

Table 13. The function of genes identified as differentially expressed by rank product statistical analysis in 2 of the 3 BL tumour lines examined.

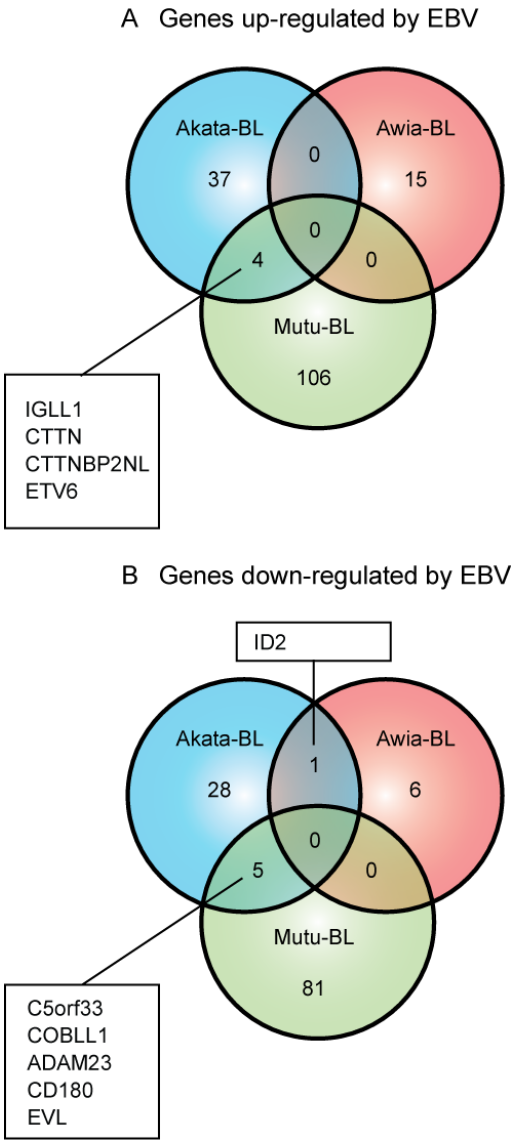


Figure 4.10. Overlap of differentially expressed genes between clones of Akata-BL, Awia-BL and Mutu-BL. (A) Overlap of genes up-regulated by EBV. (B) Overlap of genes down-regulated by EBV.

4.8.1 ID2

Inhibitor of DNA binding (ID) proteins are transcriptional regulators, which contain a helix-loop-helix (HLH) domain but lack a basic DNA-binding region (Benezra et al., 1990; Christy et al., 1991). Thus, they inhibit the functions of basic HLH transcription factors in a dominant-negative manner by suppressing binding to their heterodimerisation partners through their HLH domains (Murre et al., 1989; Sun et al., 1991). The ID2 protein negatively regulates B cell specific gene expression and B cell differentiation (Ishiguro et al., 1996; Renne et al., 2006) and has also been implicated in apoptosis sensitivity. In the osteosarcoma cell line U2OS and myeloid progenitor cell line 32D.3, ID2 expression has been shown to induce apoptosis through up-regulation of the pro-apoptotic Bcl-2 family member protein Bax (Florio et al., 1998). ID2 induced apoptosis was shown to be independent of its capacity as an inhibitor of DNA binding and was found to be mediated by ID2's N-terminal region. However, although it appears to be pro-apoptotic, ID2 has also been shown to promote proliferation in a range of cell lines (Ishiguro et al., 1995; Prabhu et al., 1997).

Figure 4.11(A) shows expression of ID2 in EBV-positive and EBV-loss clones of Akata-BL, Awia-BL, Mutu-BL and Eli-BL as determined by the GCOS software. Figure 4.11(B) and (C) show validation of ID2 expression by QRT-PCR and western blotting. As expected, we found decreased ID2 expression in EBV-positive clones of Akata-BL and Awia-BL compared to their respective EBV-positive clones using GCOS. We observed a very similar pattern of ID2 gene expression by using a commercial QRT-PCR assay for ID2; however this pattern could not be clearly discerned at the protein level. By western blotting there appeared to be no difference in ID2 expression in clones of Akata-BL and there appeared to only be a slight reduction in ID2 expression in EBV-positive Awia-BL clones. At the protein level there also appeared to be an increase in ID2 expression in EBV-positive Mutu-BL clones compared to paired EBV-loss clones. These results indicate that up-regulation of ID2 is unlikely to account for the increased sensitivity to apoptosis observed in EBV-loss BL clones.

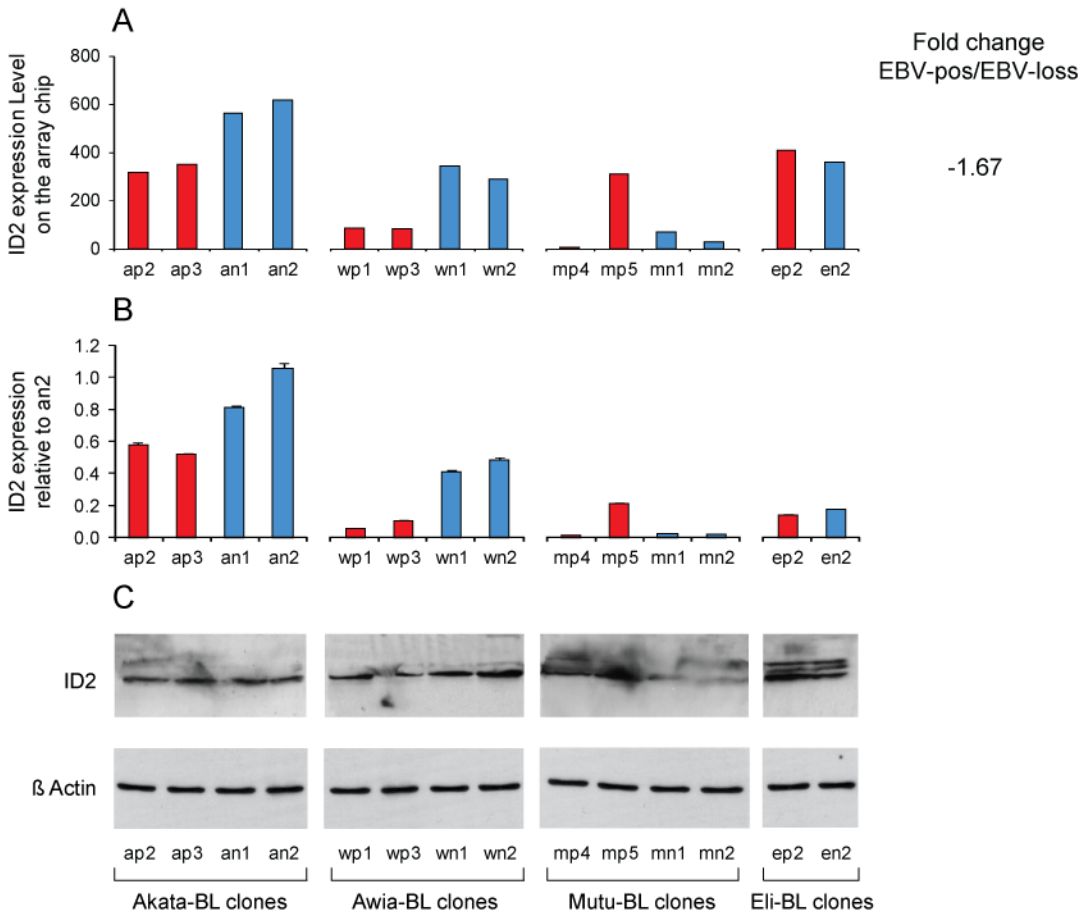


Figure 4.11. ID2 expression in EBV-positive and EBV-loss BL clones. (A) Expression data generated directly from the Affymetrix chip using GCOS. (B) ID2 expression as determined by quantitative RT-PCR and measured relative to the highest expressing clone from the Affymetrix array (an2). (C) Immunoblot probed with an antibody specific for ID2. ß Actin was used as a loading control.

4.8.2 Function of differentially expressed genes from pairwise comparisons

All the genes which are located on the microarray are assigned a function by Affymetrix based on published reports. We decided to examine the results of the pairwise comparisons in more detail by dividing genes identified as differentially expressed by EBV in clones of Akata-BL, Awia-BL and Mutu-BL into 8 functional groups (apoptosis, cell adhesion, cell cycle, motility, proliferation, signalling, transcription and translation). Figure 4.12(A) shows a heatmap of all 75 genes differentially expressed between EBV-positive and EBV-loss clones of Akata-BL. Those genes are divided into their respective function in Figure 4.12(B). It should be noted that not all differentially expressed genes fall under these 8 functional groups and many of the genes featured on the array are yet to be assigned a function. Figure 4.13 and Figure 4.14 show similar heatmaps of genes differentially expressed in clones of Awia-BL and Mutu-BL along with their function.

Within each cell line several genes with a role in apoptosis resistance were identified. The validation of some of these genes has already been shown; ID2 for example was identified as an apoptosis gene in Akata-BL and Awia-BL and was investigated in section 4.8.1. BCL2L1 (also called Bcl-XL) was identified as an apoptosis gene in clones of Mutu-BL and has been previously investigated in Chapter I, albeit using different EBV-positive clones of Mutu-BL. Additionally we investigated expression of two further apoptosis associated genes: Vascular endothelial growth factor (VEGF), which is differentially expressed in clones of Mutu-BL, and X-box binding protein 1 (XBP1), which is differentially expressed in clones of Awia-BL. VEGF promotes cell migration and inhibits apoptosis, while XBP1 is an anti-apoptotic transcription factor expressed during B cell development.

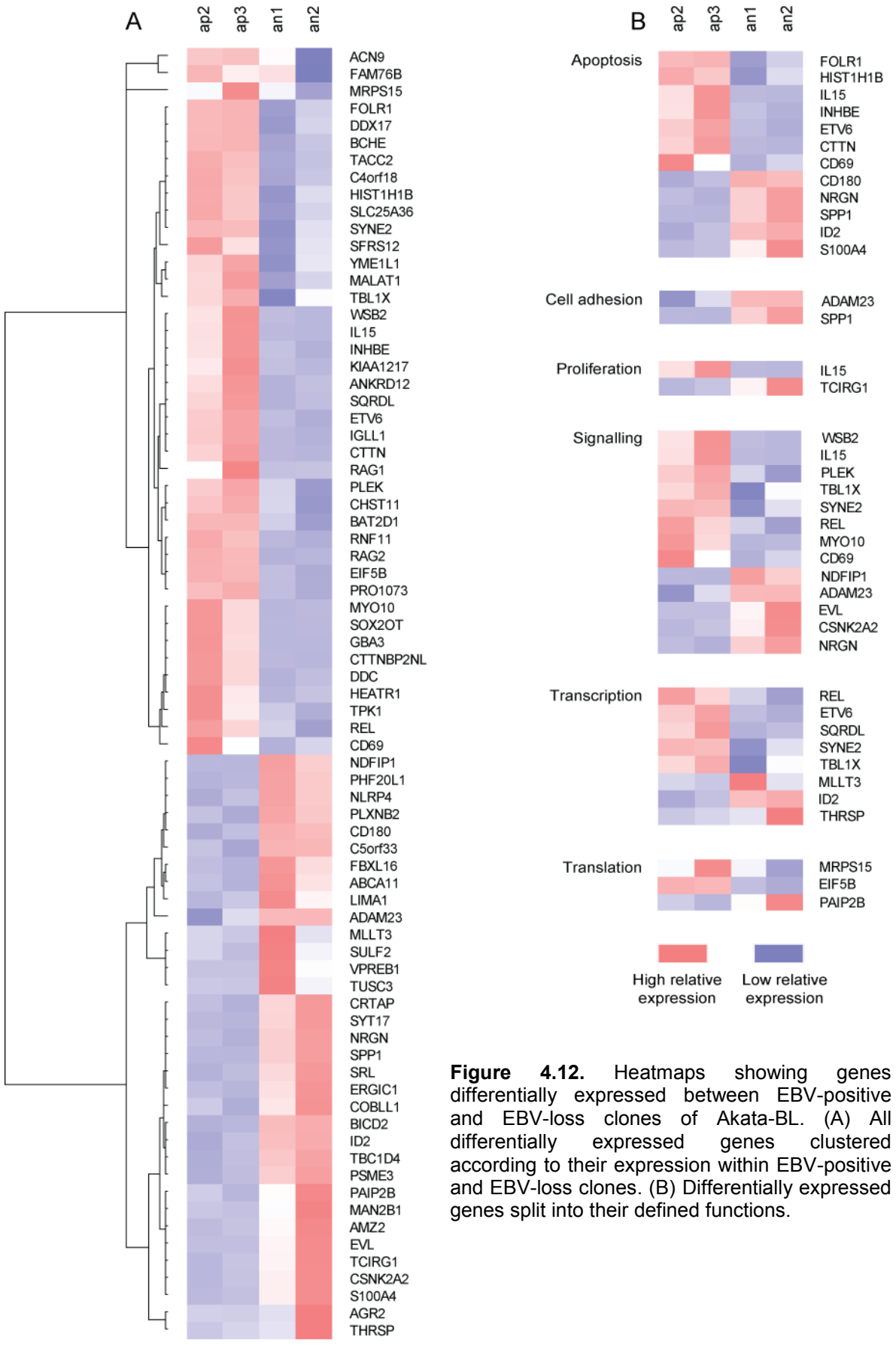


Figure 4.12. Heatmaps showing genes differentially expressed between EBV-positive and EBV-loss clones of Akata-BL. (A) All differentially expressed genes clustered according to their expression within EBV-positive and EBV-loss clones. (B) Differentially expressed genes split into their defined functions.

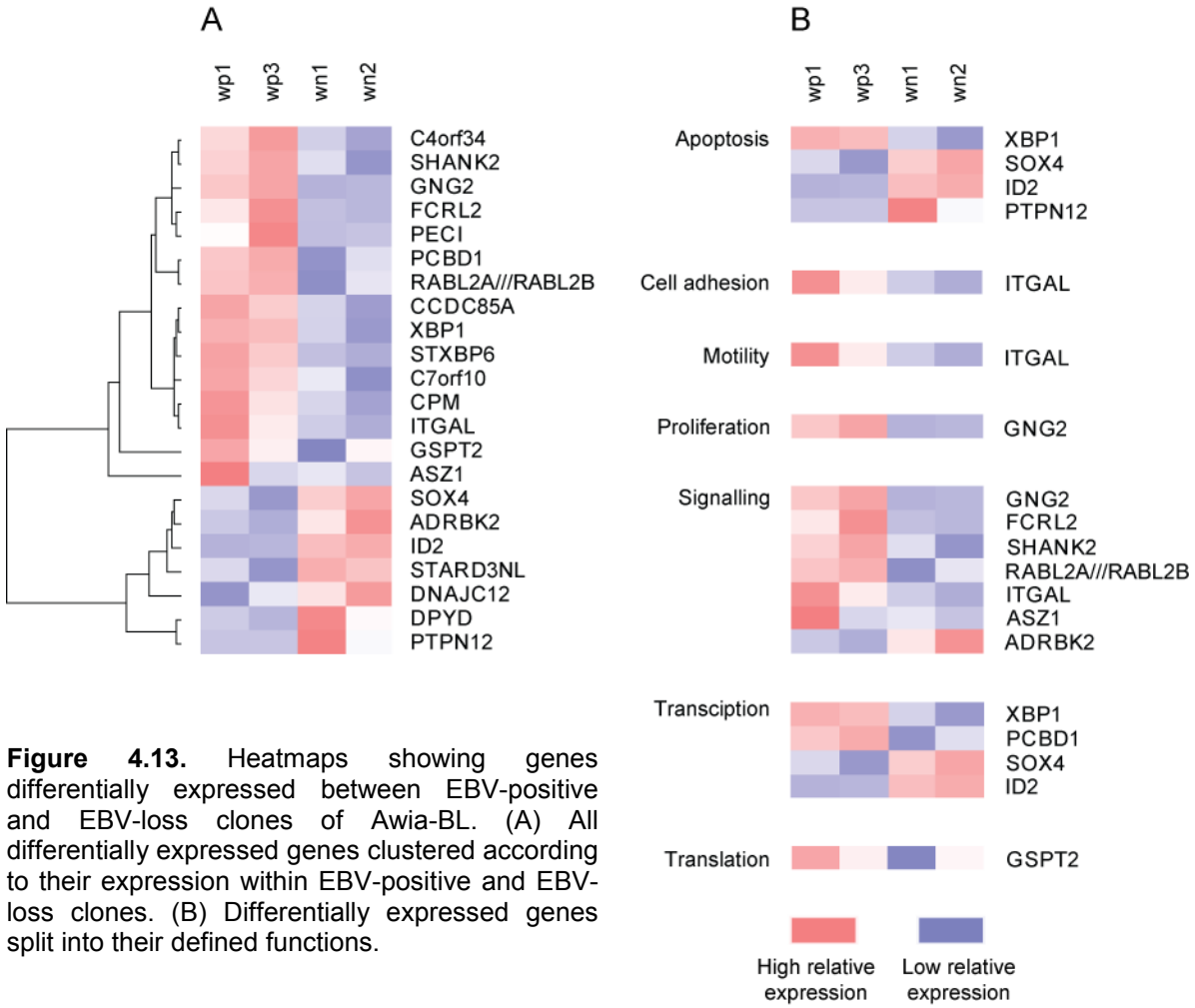
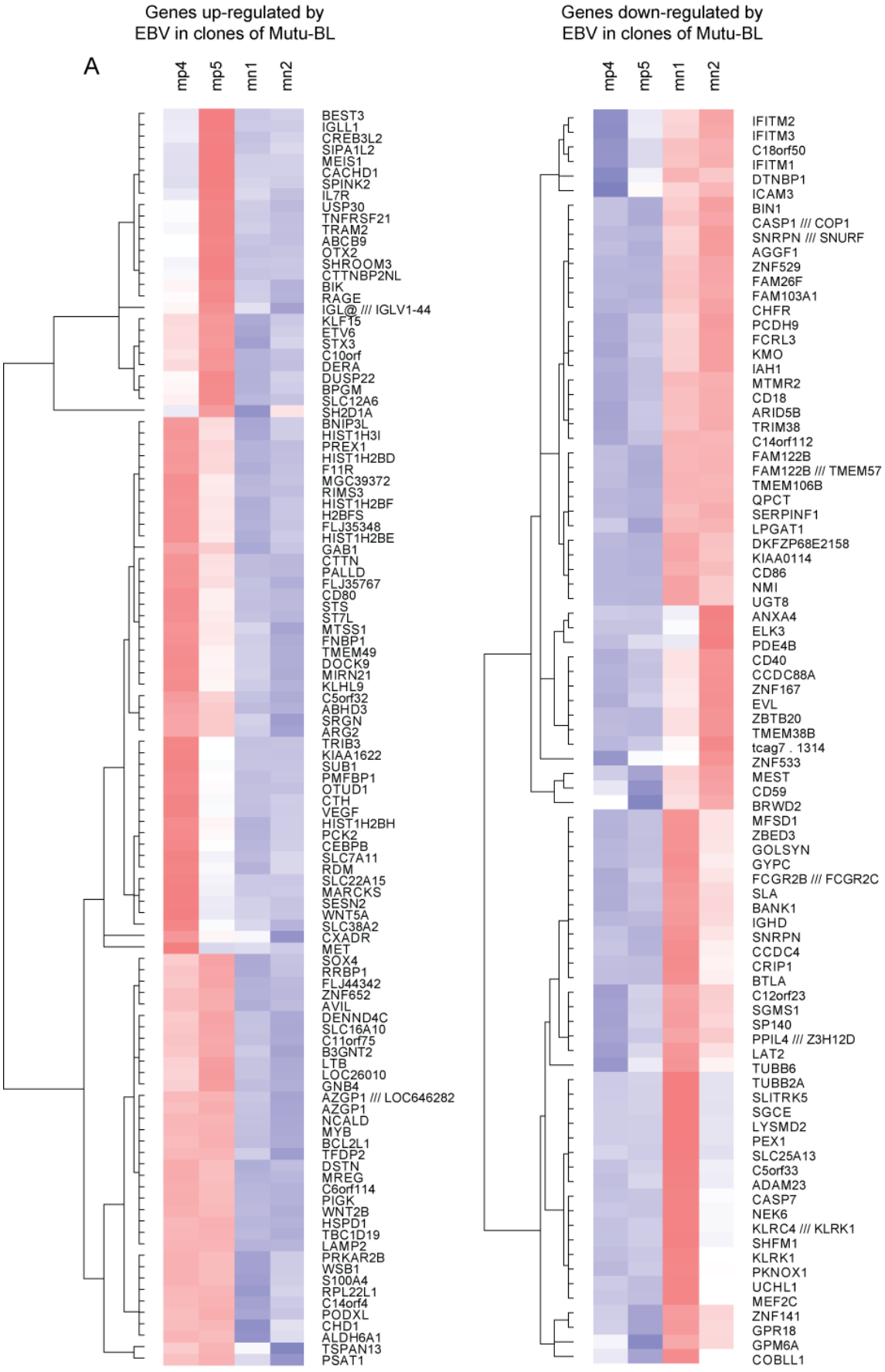


Figure 4.13. Heatmaps showing genes differentially expressed between EBV-positive and EBV-loss clones of Awia-BL. (A) All differentially expressed genes clustered according to their expression within EBV-positive and EBV-loss clones. (B) Differentially expressed genes split into their defined functions.


A
Genes up-regulated by EBV in clones of Mutu-BL
mp4
mp5
mn1
mn2
BEST3
IGLL1
CREB3L2
SIPA1L2
MEIS1
CACHD1
SPINK2
IL7R
USP30
TNFRSF21
TRAM2
ABCB9
OTX2
SHROOM3
CTTNBP2NL
BIK
RAGE
IGL@ /// IGLV1-44
KLF15
ETV6
STX3
C10orf
DERA
DUSP22
BPGM
SLC12A6
SH2D1A
BNIP3L
HIST1H3I
PREX1
HIST1H2BD
F11R
MGC39372
RIMS3
HIST1H2BF
H2BFS
FLJ35348
HIST1H2BE
GAB1
CTTN
PALLD
FLJ35767
CD80
STS
ST7L
MTSS1
FNBP1
TMEM49
DOCK9
MIRN21
KLHL9
C5orf32
ABHD3
SRGN
ARG2
TRIB3
KIAA1622
SUB1
PMFBP1
OTUD1
CTH
VEGF
HIST1H2BH
PCK2
CEBPB
SLC7A11
RDM
SLC22A15
MARCKS
SESN2
WNT5A
SLC38A2
CXADR
MET
SOX4
RRBP1
FLJ44342
ZNF652
AVIL
DENND4C
SLC16A10
C11orf75
B3GNT2
LTB
LOC26010
GNB4
AZGP1 /// LOC646282
AZGP1
NCALD
MYB
BCL2L1
TFDP2
DSTN
MREG
C6orf114
PIGK
WNT2B
HSPD1
TBC1D19
LAMP2
PRKAR2B
WSB1
S100A4
RPL22L1
C14orf4
PODXL
CHD1
ALDH6A1
TSPAN13
PSAT1
Genes down-regulated by EBV in clones of Mutu-BL
mp4
mp5
mn1
mn2
IFITM2
IFITM3
C18orf50
IFITM1
DTNBP1
ICAM3
BIN1
CASP1 /// COP1
SNRPN /// SNURF
AGGF1
ZNF529
FAM26F
FAM103A1
CHFR
PCDH9
FCRL3
KMO
IAH1
MTMR2
CD18
ARID5B
TRIM38
C14orf112
FAM122B
FAM122B /// TMEM57
TMEM106B
QPCT
SERPINF1
LPGAT1
DKFZP68E2158
KIAA0114
CD86
NMI
UGT8
ANXA4
ELK3
PDE4B
CD40
CCDC88A
ZNF167
EVL
ZBTB20
TMEM38B
tcag7 . 1314
ZNF533
MEST
CD59
BRWD2
MFSD1
ZBED3
GOLSYN
GYPC
FCGR2B /// FCGR2C
SLA
BANK1
IGHD
SNRPN
CCDC4
CRIP1
BTLA
C12orf23
SGMS1
SP140
PPIL4 /// Z3H12D
LAT2
TUBB6
TUBB2A
SLITRK5
SGCE
LYSMD2
PEX1
SLC25A13
C5orf33
ADAM23
CASP7
NEK6
KLRC4 /// KLRK1
SHFM1
KLRK1
PKNOX1
UCHL1
MEF2C
ZNF141
GPR18
GPM6A
COBLL1

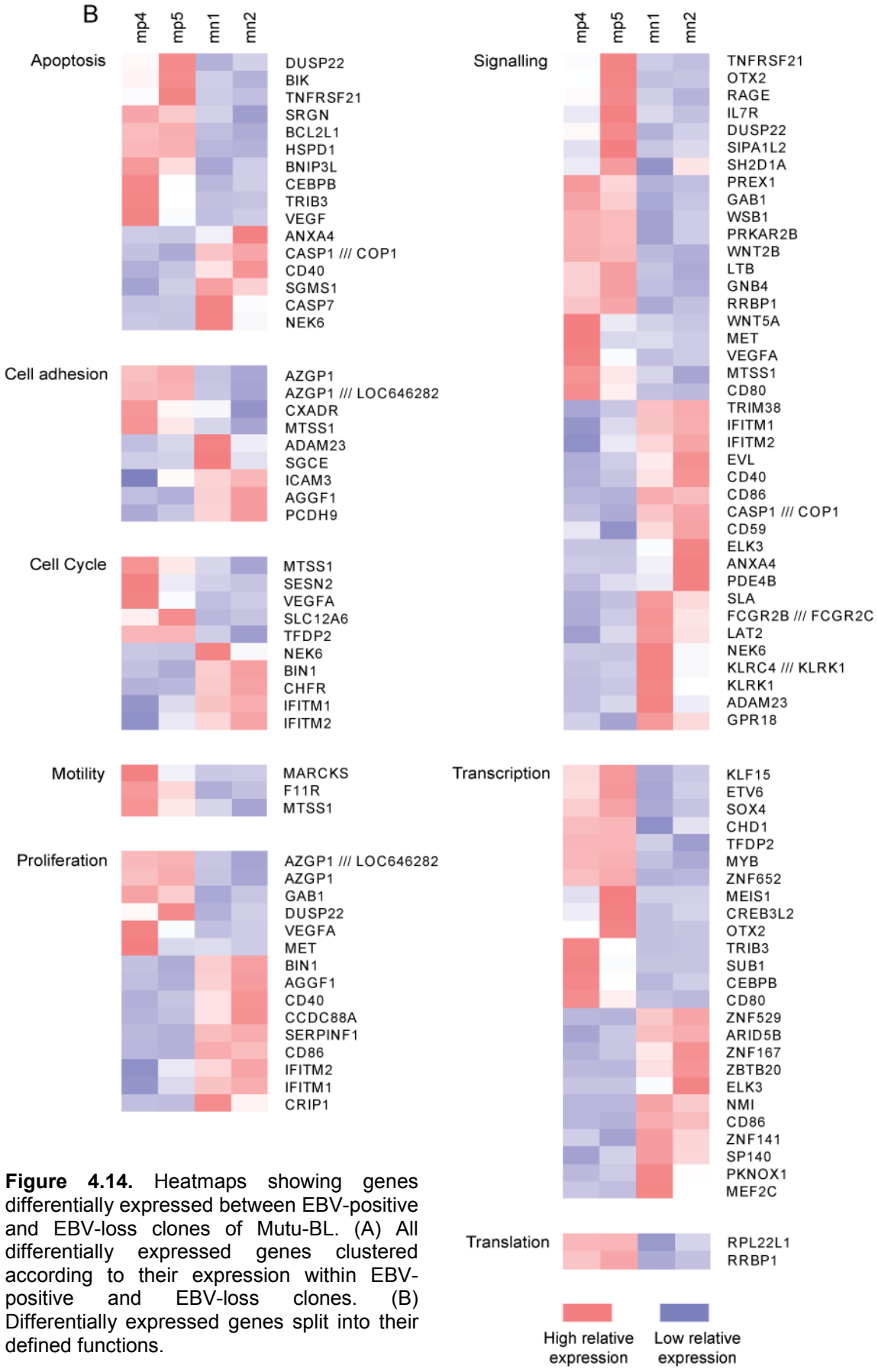


Figure 4.14. Heatmaps showing genes differentially expressed between EBV-positive and EBV-loss clones of Mutu-BL. (A) All differentially expressed genes clustered according to their expression within EBV-positive and EBV-loss clones. (B) Differentially expressed genes split into their defined functions.

The expression of VEGF and XBP1 is shown in Figure 4.15. Figure 4.15(A) shows VEGF expression data generated directly from the array using GCOS. VEGF appears to be up-regulated by at least twofold in EBV-positive clones of Mutu-BL compared to paired EBV-loss clones. However, when we investigated VEGF expression using RT-PCR (Figure 4.15(B)) we were unable to find significant VEGF expression in any EBV-positive or EBV-loss clones. This was despite clear VEGF expression in the VEGF positive controls, 293 human embryonic kidney cells and the C666.1 NPC cell line.

Figure 4.15(C) shows XBP1 expression data generated directly from the array using GCOS. XBP1 appears to be up-regulated in EBV-positive clones of Awia-BL and Mutu-BL compared to their respective EBV-loss clones. In an attempt to validate this difference in XBP1 expression we used a QRT-PCR assay (Figure 4.15(D)) to measure XBP1 expression relative to the pre-B cell line, NALM-17. Using this assay we found detectable XBP1 expression in all BL clones; however we were unable to observe the same EBV-mediated differences in gene expression as were observed using GCOS. Thus, we found once again that changes in gene expression detected by microarray analysis are not always fully representative of the steady state levels of mRNA within BL clones.

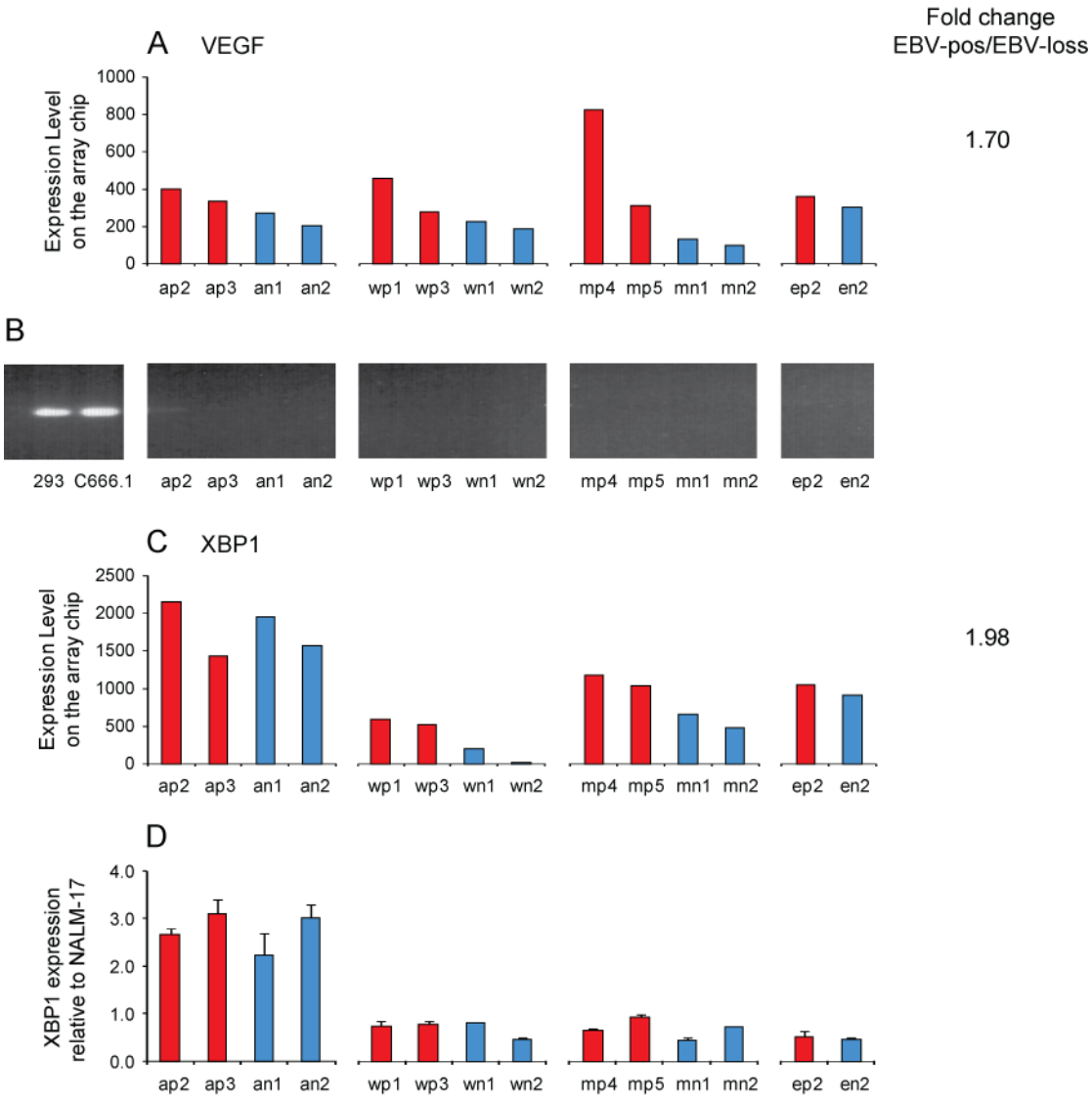


Figure 4.15. VEGF and XBP1 expression in EBV-positive and EBV-loss clones. (A) VEGF Expression data generated directly from the Affymetrix chip using GCOS. (B) VEGF expression as determined by conventional RT-PCR. (C) XBP1 expression data generated directly from the Affymetrix chip using GCOS. (D) XBP1 expression as determined by quantitative RT-PCR and measured relative to the human pre-B cell line NALM-17.

Discussion II

(a) EBV has no effect on the mBL signature

To investigate the mechanism of apoptosis protection in BL cells we used Affymetrix gene expression profiling, as this system screens the expression of all currently identified human genes. We selected representative clones from Akata-BL, Awia-BL, Mutu-BL and Eli-BL in an attempt to identify EBV-mediated changes which were broadly relevant to BL and not restricted to an individual tumour background.

The report by Hummel et al., (2006), which used a comparison of BL and DLBCL tumours to generate an mBL signature, also provided us with the opportunity to determine whether the cellular gene expression in our BL samples was typical of other BL cells. This comparison showed that cellular gene expression in BL clones closely resembled expression in mBL samples and not the DLBCL samples. In addition there was little or no difference in the expression of genes which define the mBL signature between EBV-positive and EBV-loss clones. mBL signature genes were selected on their ability to differentiate between BL and DLBCL samples where the differences in gene expression are likely to be fairly large. Thus these results are perhaps unsurprising; however it does indicate that loss of EBV does not dramatically alter cellular gene expression.

As shown in Table 9, each BL clone was assigned an index of “BL likeness”. All clones scored well above the 0.95 threshold required to be formally classified as mBL samples and several clones were in fact so similar in gene expression to the mBL signature that they were assigned an mBL index of 1. It is interesting that the mBL index of BL clones was so high, as most EBV-positive and EBV-loss clones are derived from eBL tumours, whereas the mBL signature was developed using a panel of sporadic, EBV-negative BL samples. In addition the published arrays were carried out on biopsy samples, whereas some BL clones have spent over a year in culture *in vitro*. This indicates that the global pattern of gene expression in BL cells may be determined far more by the presence of deregulated c-Myc expression than the tumour origin, the EBV status or the time spent in culture. This imposition of a gene expression phenotype by deregulated c-Myc expression has already been noticed. When investigated by gene expression profiling, BL cells were found to displayed a GC like pattern of gene expression regardless of their EBV status (Kuppers et al., 2003). In addition, LCLs

forced to overexpress myc adopted a BL like growth phenotype with a GC pattern of cell surface markers (Polack et al., 1996).

(b) Effect of EBV on cellular gene expression

The next analysis which we performed on our BL clones was to directly investigate the effect of EBV on cellular gene expression. Interestingly, despite the widespread interest in EBV's role in BL pathogenesis, a direct comparison of cellular gene expression in EBV-positive and EBV-loss clones using microarray technology has yet to be published. One study used a subtractive suppression hybridization protocol to investigate differences in gene expression in an EBV-loss clone of Akata-BL after the restoration of Latency I expression through reinfection with a recombinant EBV (Kiss et al., 2003). However this method lacks the sensitivity of the Affymetrix array systems and the authors were able to identify only a small number of cellular genes which were affected by EBV infection. Of these cellular genes, expression of only one (Tcl-1) could explain the observed differences in apoptosis resistance between EBV-positive and EBV-loss clones; however our data indicates that expression of this gene is unaffected by EBV status.

In the present study we used rank product statistical analysis to screen for novel genes which may be regulated by EBV in BL clones. Rank product was chosen over conventional statistical methodologies such as significance analysis of microarrays (SAM) because of its ability to more effectively identify genes with the greatest fold changes (FC) in their expression (Breitling et al., 2004; Breitling and Herzyk, 2005; Hong and Breitling, 2008). Rank product is also able to identify these more biologically relevant large FC genes with fewer replicates, which is key, given the small number of EBV-positive and EBV-loss clones from each BL background in this study.

(i) Analysis of differentially expressed genes

Using rank product we identified 444 genes which were differentially expressed between EBV-positive and EBV-loss clones, expression of which we viewed using heatmaps. Within these heatmaps we clustered BL clones first by EBV status (supervised clustering), then by their expression of the 444 differentially expressed genes (unsupervised clustering) (Figure 4.3 and Figure 4.4). Interestingly, these clustering analyses revealed that there was a greater difference in gene expression between

clones from different tumour backgrounds than between EBV-positive and EBV-loss clones from the same background. This was despite the fact that the genes which were used in the clustering analysis were pre-selected because of a differential expression between EBV-positive and EBV-loss clones. Presumably this effect would have been even more noticeable if genes were included in the clustering analysis which had not been identified as being differentially expressed between EBV-positive and EBV-loss clones.

The results indicate that, although all the BL clones retain an mBL signature, there may still be significant differences in the gene expression between the different BL backgrounds. This is perhaps to be expected as there are a number of fundamental differences between the different cell lines. For example, all of the BL tumours are derived from different BL patients and while most were eBL tumours, Akata-BL is derived from a sporadic BL sample. Each cell line carries the common 8:14 c-myc translocation (Kelly et al., 2006; Rowe et al., 1985; Takada et al., 1991); however the tumours from which these cell lines are derived presented at different sites ranging from the jaw to the abdomen and ovaries. It is also likely that each tumour independently acquired additional mutations to counteract the high level of apoptosis induced by deregulated c-myc expression. In addition to these mutations acquired *in vivo* each of the cell lines has spent a variable amount of time in culture *in vitro*, during which cells may have been selected for advantageous growth mutations.

To examine expression of the 444 differentially expressed genes in more detail, we generated bar charts of gene expression for each gene and found that no genes were differentially expressed between EBV-positive and EBV-loss clones in all 4 cell backgrounds. This is a surprising observation as transcriptional regulation has been demonstrated by other herpes viruses during latent infections. For example endothelial cells latently infected with Kaposi's sarcoma-associated herpesvirus (KSHV) up-regulated a range of interferon-responsive genes (Poole et al., 2002), while neuronal cells quiescently infected with herpes simplex virus type 1 (HSV-1) showed significant modulation of genes involved in growth, metabolism and apoptosis (Danaher et al., 2008). In addition EBNA1 has been shown to modulate gene expression in both HL (Flavell et al., 2008) and NPC cells (O'Neil et al., 2008); however this effect has not been demonstrated in a BL background.

Despite the fact that no genes appeared to be universally regulated by EBV, there were several genes which appeared to be differentially expressed in the majority of cell backgrounds. Three of these

genes (TNFAIP3, NMI and CD86) also had published roles in apoptosis, hence we analysed their mRNA levels by PCR and their protein levels by immunoblotting or cell surface staining. The PCR results broadly correlated to the data generated from the array; however at the protein level we found little or no difference in expression of these genes between EBV-positive and EBV-loss clones. The disparity between the results of microarray and RT-PCR analysis and the results of protein expression studies could be due to production of protein from mRNA transcripts not detected by gene expression profiling or RT-PCR or because of differences in turnover of protein in different clones. Alternatively the roughly 2-fold differences observed by gene expression profiling may be too small to translate into meaningful differences in protein expression.

As we could find no differences in gene expression which were consistent across multiple BL backgrounds, we reasoned that EBV may be affecting different genes in each BL tumour. We investigated this possibility by generating lists of differentially expressed genes in the individual BL cell lines, Akata-BL, Awia-BL and Mutu-BL. In accordance with our previous observations, no genes appeared in all three independent lists and only 10 genes overlapped 2 of the 3 BL backgrounds (Figure 4.10). When we examined the published function of these 10 genes (Table 13), we identified only one (ID2) whose expression might be able to explain the phenotype of EBV-positive clones; however no difference in ID2 protein expression could be found between EBV-positive and EBV-loss clones. By dividing the lists of differentially expressed genes into function we also identified XBP1 and VEGF as apoptosis-related genes differentially expressed in a single background; however mRNA levels of these genes did not match those predicted by gene expression profiling. This could be due to detection of different splice variants by differently located primers in the array and the PCR reaction and demonstrates once again that small changes observed by gene expression profiling are difficult to validate.

The inability to find reproducible differences in gene expression between EBV-positive and EBV-loss clones could be responsible for the lack of published gene expression profiling data on EBV-positive and EBV-loss clones. It is possible that this technique is more suited for identifying differences between less closely related cell populations, such as the difference between the BL and either the DLBCLs (Dave et al., 2006; Hummel et al., 2006) or the LCL (Schlee et al., 2007) phenotypes.

(ii) Alternative explanations for the observed patterns of cellular gene expression

There are several possible scenarios that could explain the apparent lack of common changes in gene expression between EBV-positive and EBV-loss clones. The first is that different genes are regulated by EBV in the different BL cell lines; i.e. c-myc translocation-positive tumours may evolve to a broadly similar end point but by different routes In other words, EBV may target different pathways or different points on the same pathway in different BL tumours. As observed in the previous chapter, Akata-BL is p53 null whereas the other cell lines have a mutated non-functional p53. Still more BL cell lines have been found with WT p53, but with an overexpression of MDM2 (Lindstrom et al., 2001). In this manner all clones could retain an mBL signature, while at the same time having a number of differentially expressed genes. If the experiment had been simplified by using multiple clones from only one background there would have been a greater probability of identifying differentially expressed genes; however it is unlikely that these changes would be meaningful in the wider context of BL pathogenesis if they could not be replicated in several BL backgrounds. Another more straightforward experiment frequently used in gene expression profiling is transfection of a single viral product into a virus-negative background followed by microarray analysis (O'Neil et al., 2008). It would have been possible to transfect individual Latency I gene products into EBV-loss clones; however at this point we had not assayed the protective abilities of the individual viral gene products and this method would suffer from the same issues of biological relevance as investigation of only a single BL cell line.

Another possibility is that rank product statistical analysis was simply not a powerful enough approach with which to investigate this problem. The comparison of BL clones from different BL backgrounds is a fairly complicated statistical problem and we noticed during analysis of the data that rank product in several cases failed to produce meaningful results from the noisy biological data. Although rank product was selected on the basis that it can identify the genes with the greatest FC between BL clones, many of the identified genes had a low FC between EBV-positive and EBV-loss clones and on closer inspection were in fact differentially expressed in only one cell background or even in only a single clone. There were also several instances where a gene was up-regulated in EBV-positive clones of one cell background, but down-regulated in EBV-positive clones of another. Several new

statistical approaches such as gene enrichment analysis have been developed which incorporate current understanding of the biological function of the genes represented on the microarray (Song and Black, 2008; Subramanian et al., 2005). However rank product is still considered to be an effective option to deal with noisy biological data sets, especially those which have small numbers of replicates (Jeffery et al., 2006). The statistical rigour of this experiment could have been improved if more EBV-positive and EBV-loss clones from each cell background had been included in the analysis; however the prohibitive cost of such experiments meant that, with available funds, the choice of clones was the most cost effective way to investigate gene expression in multiple cell backgrounds.

It is also possible that EBV mediated changes in cell phenotype are not apparent in the steady state mRNA level. EBV latent gene products could cause a difference in the splicing of target genes, which would not be detected using Affymetrix as this technique analyzes expression of only the 3' end of cellular mRNA transcripts. Recently, exon arrays have been developed which have multiple probes per exon of each gene enabling expression of different isoforms of a gene to be investigated as well as their overall expression. Interpretation of the results of these arrays would be problematic as the statistical analysis would become even more complex.

Alternatively EBV-mediated changes in gene expression could be apparent only after treatment with an apoptotic stimulus. This possibility would be very difficult to analyse using gene expression profiling as the Affymetrix protocol requires large quantities of high quality mRNA, which may be degraded by the induction of apoptosis. In addition it would be impossible to say whether changes in gene expression were causal or simply representative of the differing levels of apoptosis in EBV-positive and EBV-loss clones.

A final possibility is that Latency I gene products exert their effects in a post-translational manner. This could be through a direct protein-protein interaction by EBNA1 or possibly a BART encoded protein, an RNA-protein interaction by the EBERs, an inhibition of mRNA processing by the EBV-encoded microRNAs or through a yet to be fully characterised mechanism; for example, low level expression of a lytic cycle antigen, such as vIL-10. Several of these possibilities are investigated in the following chapter.

5. Results Part III

Restoration of Latency I viral transcripts to EBV-loss clones

5.1 Introduction

The aims of this part of the study are twofold: firstly we attempted to formally show that the apoptosis resistance observed in EBV-positive clones is virus-mediated and secondly we investigated which of the EBV transcripts found in Latency I is responsible for this apoptosis protection.

5.2 Reinfection of EBV-loss clones with recombinant EBV

To formally demonstrate that the increased resistance to apoptosis in EBV-positive clones was due to the presence of EBV, it was essential to restore Latency I viral gene expression to EBV-loss clones. In previous investigations, infection of EBV-loss clones with recombinant EBV resulted in the same restricted Latency I pattern of viral gene expression observed in EBV-positive clones and restored the phenotype associated with EBV positivity (Komano et al., 1998; Ruf et al., 1999).

In this study, EBV-loss cells were either reinfected with the 2089 EBV strain developed by Henri-Jacque Delecluse or Akata virus generated from EBV-positive Akata-BL cells by Kenzo Takada. Generation of 2089 and Akata virus is described in more detail in section 2.16.1. Briefly, 2089 is based on the B95-8 virus strain, but contains a constitutively active green fluorescence protein (GFP) gene and hygromycin resistance gene inserted adjacent to the B95-8 11.8kb deleted region (Delecluse et al., 1998). Recombinant Akata virus has a constitutively active GFP and neomycin resistance cassette, which replaces the non-essential EBV thymidine kinase (TK) gene (Shimizu et al., 1996). During the investigation into the effect of reinfection of EBV-loss clones with EBV we used 2 different infection methods; initially we attempted to isolate Latency I reinfected cells by using the GFP marker of infected cells, but we also used a second method which utilised the antibiotic resistance cassettes within the recombinant viruses.

5.2.1 Establishment of reinfected populations by single cell sorting

In a preliminary experiment, we determined which EBV-loss BL clones were most infectable with EBV by simply incubating EBV-loss clones from Akata-BL, Awia-BL, Mutu-BL and Eli-BL with 2089 or Akata virus and screening after 48 hours for GFP-positive cells by flow cytometry. Using this method, Awia-BL and Mutu-BL cells were found to be the most easily infected with recombinant EBV. We also found there was no difference in the infectability between 2089 and Akata virus in EBV-loss BL clones (data not shown).

We then attempted to establish a latent infection using the method outlined in Figure 5.1. $1x10^6$ EBV-loss cells of Awia-BL or Mutu-BL were incubated with $50x10^6$ 2089 or Akata virus particles as described in section 2.16.4. Infected cells were then cultured for 48 hours to allow production of virally encoded GFP. Single GFP-positive cells were then sorted into wells of 96 well plates pre-seeded with $2x10^3$ human fibroblast cells and cultured for 4 weeks. Once a pellet of cells had formed, half the cells were harvested for DNA extraction. Quantitative DNA-PCR was then used to determine the viral load in each clone and QRT-PCR was used to determine viral latency.

The rationale for using this methodology was threefold. When working with BL cell lines such as Mutu-BL, we have previously observed that a small number of Latency III cells within a culture can quickly result in the conversion of the entire culture to Latency III; thus, we believed that we could maximise the probability of isolating Latency I infection by generating cultures from single infected cells. Additionally, we wanted to establish a panel of infected clones from the infection of each EBV-loss clone and we wished to avoid drug selection as it is possible that artificial selection of cells with antibiotics can promote the outgrowth of apoptosis resistant cells.

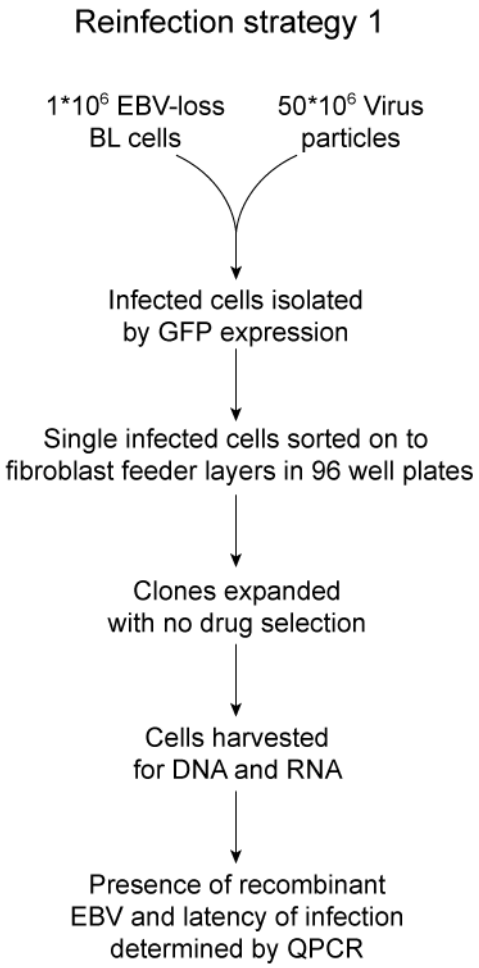


Figure 5.1 Flow chart of the initial strategy used to infect EBV-loss clones with recombinant EBV.

5.2.1.1 Results from reinfection using single cell sorting

The results from 2 representative reinfection experiments (one with an Awia-BL clone and the other with a Mutu-BL clone) are shown in Figure 5.2. Single cell sorting of infected Awia-BL clones on to fibroblast feeder layers generated 37 infected clones. When these were analysed by quantitative DNA-PCR for the EBV pol gene, 15 of the 37 clones were either completely EBV-negative or had less than 1 copy of EBV per 100 cells. Of the 22 remaining clones, only 6 had an average viral load of greater than 0.5 genomes per cell. When reinfected clones of Mutu-BL were analysed, only 4 clones had an average viral load of greater than 0.5 viral genomes per cell. This indicates that, without drug selection, EBV is rapidly lost from reinfected EBV-loss BL cells.

It has previously been reported that infection of EBV-loss clones of Akata-BL and Mutu-BL leads to a return to the restricted Latency I viral gene expression observed in BL tumours and not the full Latency III infection seen in infection of primary B cells (Komano et al., 1999; Trivedi et al., 2001). We therefore examined EBV latent gene expression in the 10 EBV-loss clones (6 Awia-BL clones and 4 Mutu-BL clones) which retained more than 0.5 EBV genomes per cell. RNA was extracted from aliquots of the reinfected clones and reverse transcribed into cDNA using gene specific primers. We used specific QRT-PCR assays to investigate activity of the EBV latent promoters Qp, Cp and Wp; we examined expression of the EBV latent transcripts EBNA2 and LMP1 and activity of the EBV lytic cycle via activation of the lytic F promoter (Fp) and expression of the immediate early lytic cycle gene, BZLF1.

The results of QRT-PCR analysis of reinfected clones is shown in Figure 5.3. We found no Qp derived Q-U-K transcripts in any of the reinfected clones investigated. Instead we found LCL-like expression of Cp and Wp transcripts along with detectable levels of the major growth transforming proteins EBNA2 and LMP1. Thus, reinfection of these EBV-loss clones has resulted in a Latency III pattern of virus gene expression. Viral expression in reinfected clones also appeared to be tightly latent as no F-U or BZLF1 transcripts were detected.

Overall, the results of infection strategy 1 show that: i) without drug selection EBV is rapidly lost from reinfected EBV-loss clones and ii) most if not all reinfections result in expression of the full Latency III growth transforming programme. We therefore decided to use drug selection as a means of establishing a greater number of reinfected clones, with the hope that this would increase the probability of isolating a rare Latency I reinfected clone.

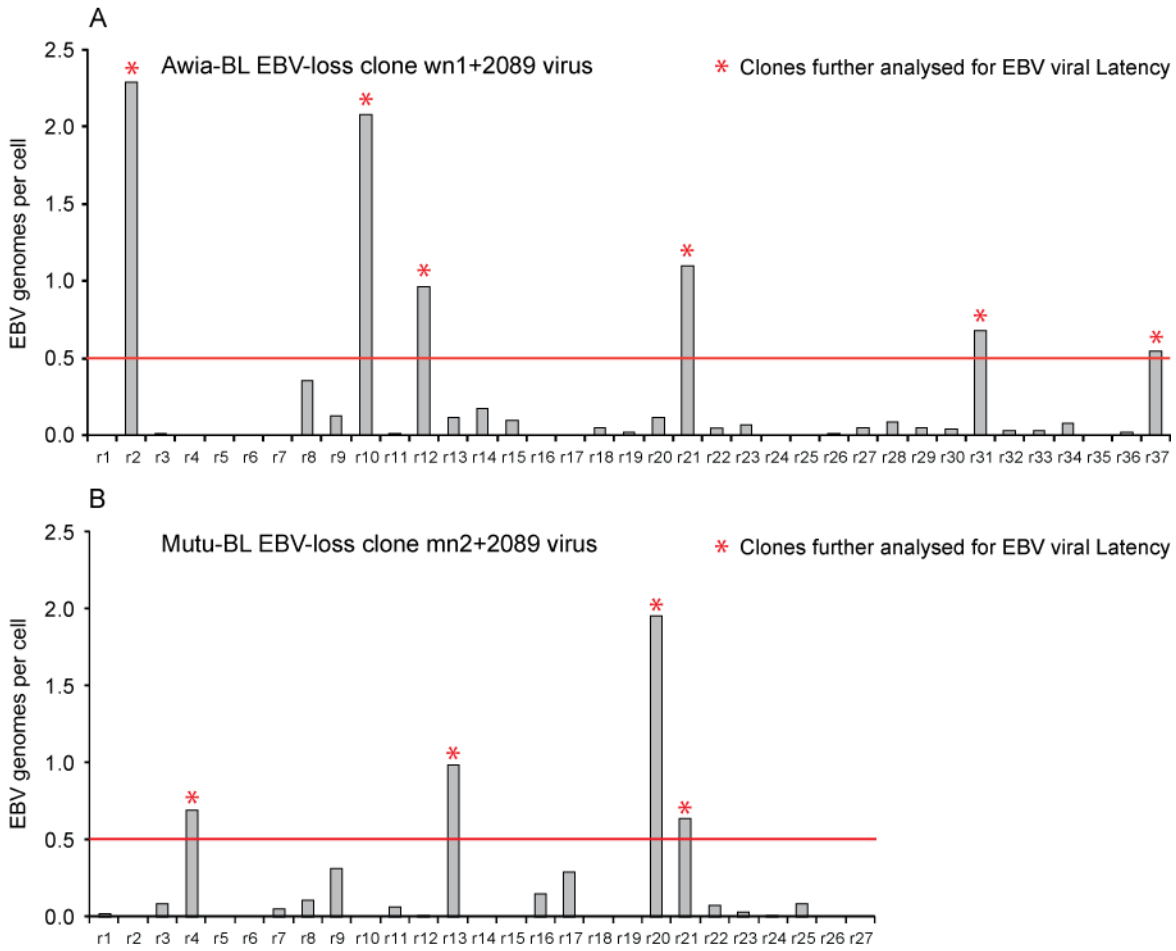
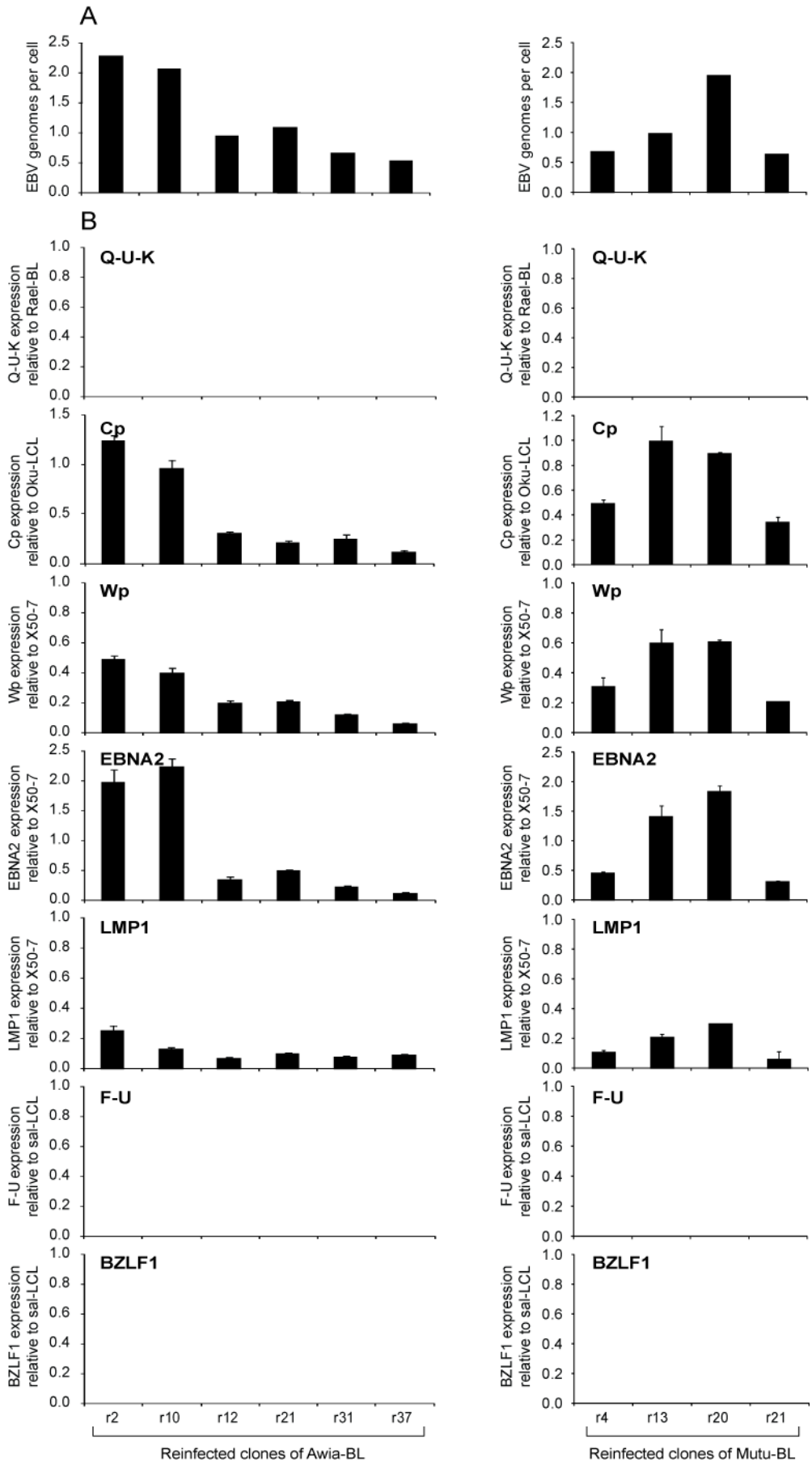


Figure 5.2 Viral load of single cell reinfected clones. (A) Awia-BL EBV-loss clone wn1 reinfected with 2089 virus. (B) Mutu-BL EBV-loss clone mn2 reinfected with 2089 virus. EBV viral load was determined by quantitative DNA-PCR for the EBV pol gene. Clones with an average EBV viral load greater than 0.5 genomes per cell (as indicated by the red threshold line) were selected for further analysis of EBV viral latency.

Figure 5.3. (next page) EBV gene expression in single cell reinfected clones of EBV-loss Awia-BL and EBV-loss Mutu-BL. (A) EBV viral load in selected EBV reinfected clones of Awia-BL (left panel) and Mutu-BL (right panel) as determined by quantitative DNA-PCR. This data was previously shown in Figure 5.2, but is shown again as reference for the remaining figure. (B) EBV gene expression in EBV reinfected clones of Awia-BL (left panel) and Mutu-BL (right panel) as determined by QRT-PCR. Transcription levels were measured relative to an appropriate positive control (assigned a value of 1). Error bars indicate the range between duplicate assays carried out in parallel on the same cDNA sample.


A
EBV genomes per cell
B
Q-U-K
Q-U-K expression relative to Rael-BL
Cp
Cp expression relative to Oku-LCL
Wp
Wp expression relative to X50-7
EBNA2
EBNA2 expression relative to X50-7
LMP1
LMP1 expression relative to X50-7
F-U
F-U expression relative to sal-LCL
BZLF1
BZLF1 expression relative to sal-LCL
r2
r10
r12
r21
r31
r37
Reinfected clones of Awia-BL
r4
r13
r20
r21
Reinfected clones of Mutu-BL

5.2.2 Establishment of reinfected populations using drug selection

To increase the probability that a rare Latency I reinfection would be retained we decided to use an alternative reinfection strategy, which utilised the hygromycin resistance marker present in 2089 virus and the neomycin resistance marker present in Akata virus. In a preliminary experiment, the minimum concentration of hygromycin and neomycin (G418) required to induce cell death was titrated for each of the EBV-loss BL clones. EBV-loss clones were then infected using the method outlined in Figure 5.4. As for reinfection strategy 1, $1x10^6$ EBV-loss cells were incubated overnight with $50x10^6$ 2089 or Akata virus particles as described in 2.16.4. After 24 hours, cells were transferred to medium containing the appropriate antibiotic concentration and the antibiotic supplemented medium was replaced twice weekly until a drug resistant population had grown through. 2 independent attempts were made to infect each of the 3 EBV-loss clones of Akata-BL, 2 EBV-loss clones of Awia-BL, 3 EBV-loss clones of Eli-BL and 3 EBV-loss clones of Mutu-BL using 2089 and 2 further attempts were made using Akata virus. Once antibiotic resistant populations had grown through, cells were harvested for DNA and RNA extraction.

5.2.2.1 Results from reinfection using drug selection

No antibiotic resistant lines could be generated from EBV-loss clones of Akata-BL indicating an inability of EBV to establish a persistent infection in these cells. EBV-loss clones from other BL cell lines were however much more amenable to EBV infection. In several independent experiments, we successfully infected multiple cultures of EBV-loss Awia-BL, Eli-BL and Mutu-BL with both 2089 and Akata virus. DNA was extracted from these bulk cultures and EBV viral load was determined using quantitative DNA-PCR.

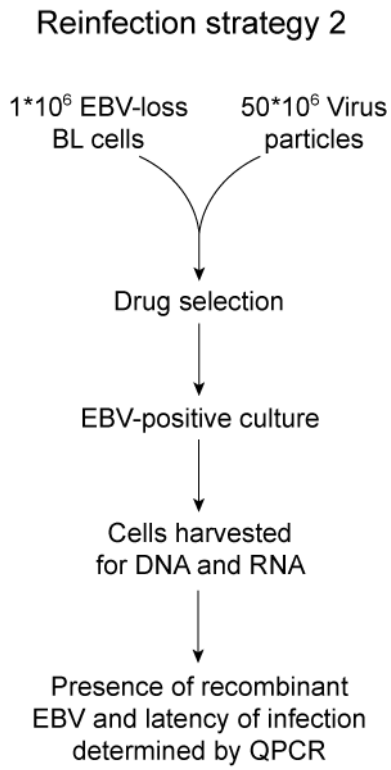


Figure 5.4. Flow chart of the second strategy used to infect EBV-loss clones with recombinant EBV

Representative data from the infection of 2 EBV-loss clones of Awia-BL, 2 EBV-loss clones of Eli-BL and 4 EBV-loss clones of Mutu-BL is shown in Figure 5.5(A). Drug resistant bulk culture infections were all found to be EBV-positive and we noticed that after drug selection, EBV genome load was higher than the reinfected clones generated using strategy 1.

RNA extracted from reinfected bulk cultures was reverse transcribed to cDNA using EBV gene specific primers and EBV viral latency was determined using QRT-PCR (Figure 5.5(B)). Specific QRT-PCR assays were used to investigate activity of the EBV latent promoters Qp, Cp and Wp, expression of the EBV latent transcripts EBNA2 and LMP1 and activity of the EBV lytic cycle via activation of Fp and expression of the immediate early lytic cycle gene, BZLF1. We found that the vast majority of bulk culture infections displayed a Latency III pattern of viral gene expression characterised by significant expression from Cp and Wp and LCL like levels of the major growth transforming proteins, EBNA2 and LMP1. By contrast, they were negative or had only very low expression of Qp derived Q-U-K transcripts. EBV gene expression in these reinfected clones was also found to be tightly latent, as there were only barely detectable levels of the lytic F-U or BZLF1 transcripts.

The single exception to this pattern of gene expression was an EBV-loss clone of Mutu-BL (mn3) reinfected with recombinant Akata virus. This reinfected clone had levels of Q-U-K expression of around 10% of that observed in the reference Latency I cell line, Rael-BL, which was not an artefact of lytic Fp expression as this promoter was silent. This low level Qp expression was however accompanied by low level expression from Cp, traces of Wp derived transcripts and EBNA2 and LMP1 transcripts at around 20% of the level observed in X50-7 LCL. Expression of transcripts from both Qp and Cp/Wp indicated that this clone contained a mixture of cells, some with a restricted Latency I infection and others with full Latency III viral gene expression. Interestingly the viral load of this mixed population was also higher than any other reinfected clone investigated. This increased viral load was not due to lytic activity, as both F-U and BZLF1 transcripts were absent from this clone. To determine if a pure EBV reinfected Latency I cell population could be isolated from this bulk culture, cells were single cell cloned by limiting dilution as described below.

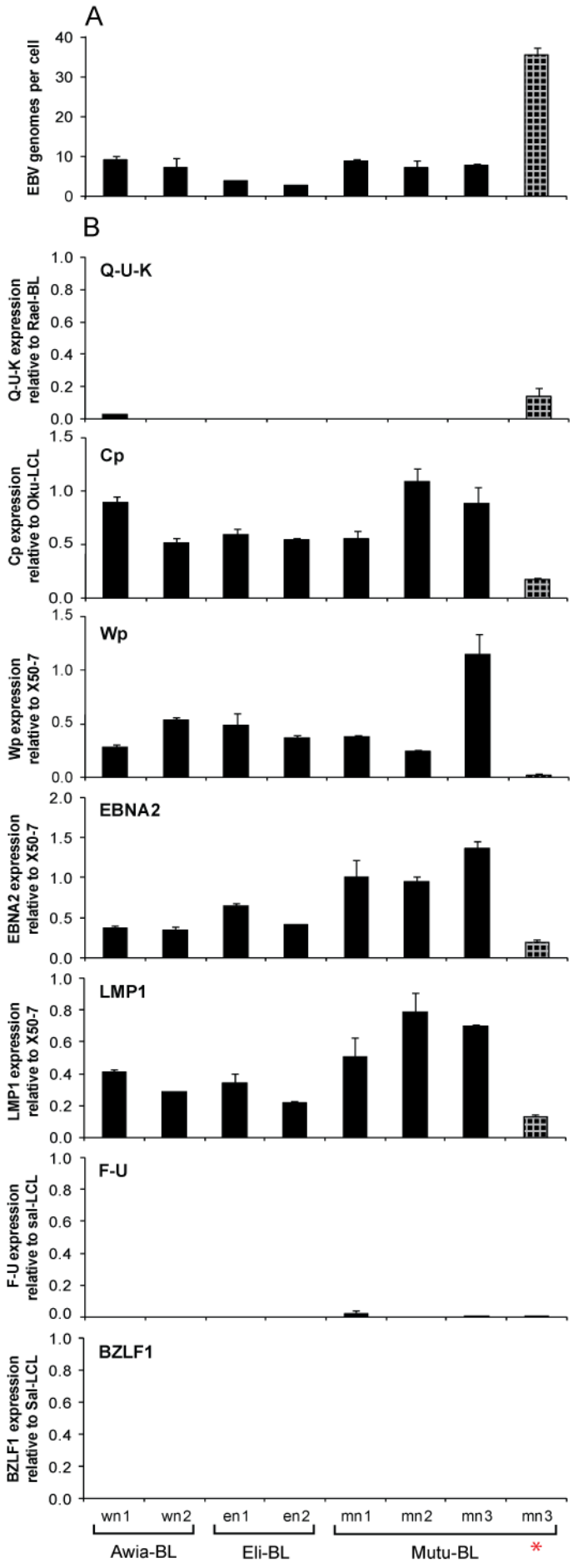


Figure 5.5. QPCR analysis of bulk culture infections of EBV-loss clones of Awia-BL, Eli-BL and Mutu-BL. (A) EBV viral load in reinfected clones as determined by quantitative DNA-PCR for the EBV pol gene. (B) EBV gene expression as determined by QRT-PCR. Transcription levels were measured relative to an appropriate control (assigned a value of 1). Error bars indicate the range between duplicate assays carried out in parallel on the same cDNA sample. The * indicates the single infection that resulted in a mixture of Latency I and Latency III viral gene expression.

5.2.3 Single cell cloning of a mixed bulk culture infection

In an attempt to isolate reinfected cells with Latency I viral gene expression, the bulk culture which expressed both Latency I and Latency III transcripts (mn3+Akata virus) was single cell cloned by limiting dilution on to human fibroblast feeder layers in wells of 96 well plates. For the first 7 days clones were grown in normal medium to allow clones to establish themselves and to increase the longevity of the fibroblast feeder layers, which are not antibiotic resistant. Neomycin (G418) was then added to the medium to prevent loss of EBV from mn3+Akata virus subclones. Single cell cloning of parental BL cell lines in the absence of drug selection (section 3.2) normally generated between 75 and 150 clones from 3 96 well plates; however only 28 reinfected clones were isolated from 3 96 well plates in this cloning experiment. This reduction is likely due to reduced viability of fibroblast feeder layers after the introduction of G418 to the medium. DNA was extracted from these 28 clones and the EBV viral load was examined by quantitative DNA-PCR.

As shown in Figure 5.6(A), all mn3+Akata virus subclones were EBV-positive and the EBV viral load varied from an average of 15 to 94 EBV genomes per cell. As would be expected from the expression of both Latency I and Latency III transcripts in the parental cell line, some of the clones displayed the single cell appearance of Latency I or EBV-loss BL cells while others had the clumpy appearance characteristic of Latency III viral gene expression. As we were interested in Latency I reinfections, 6 single cell clones with a range of viral loads were selected for further analysis. To ensure that every cell within the infected cultures was harbouring EBV, we analysed the percentage of EBER positive cells using flow cytometry. As shown in Figure 5.6(B), EBERs were found in around 80% of the cells from clones r1, r2, r3, r4 and r6, which is equivalent to the staining observed in Raji-BL where every cell has been shown to harbour around 50 copies of EBV. Clone r5, however, had slightly lower EBER staining possibly indicating that it contained a subpopulation of EBV-negative cells.

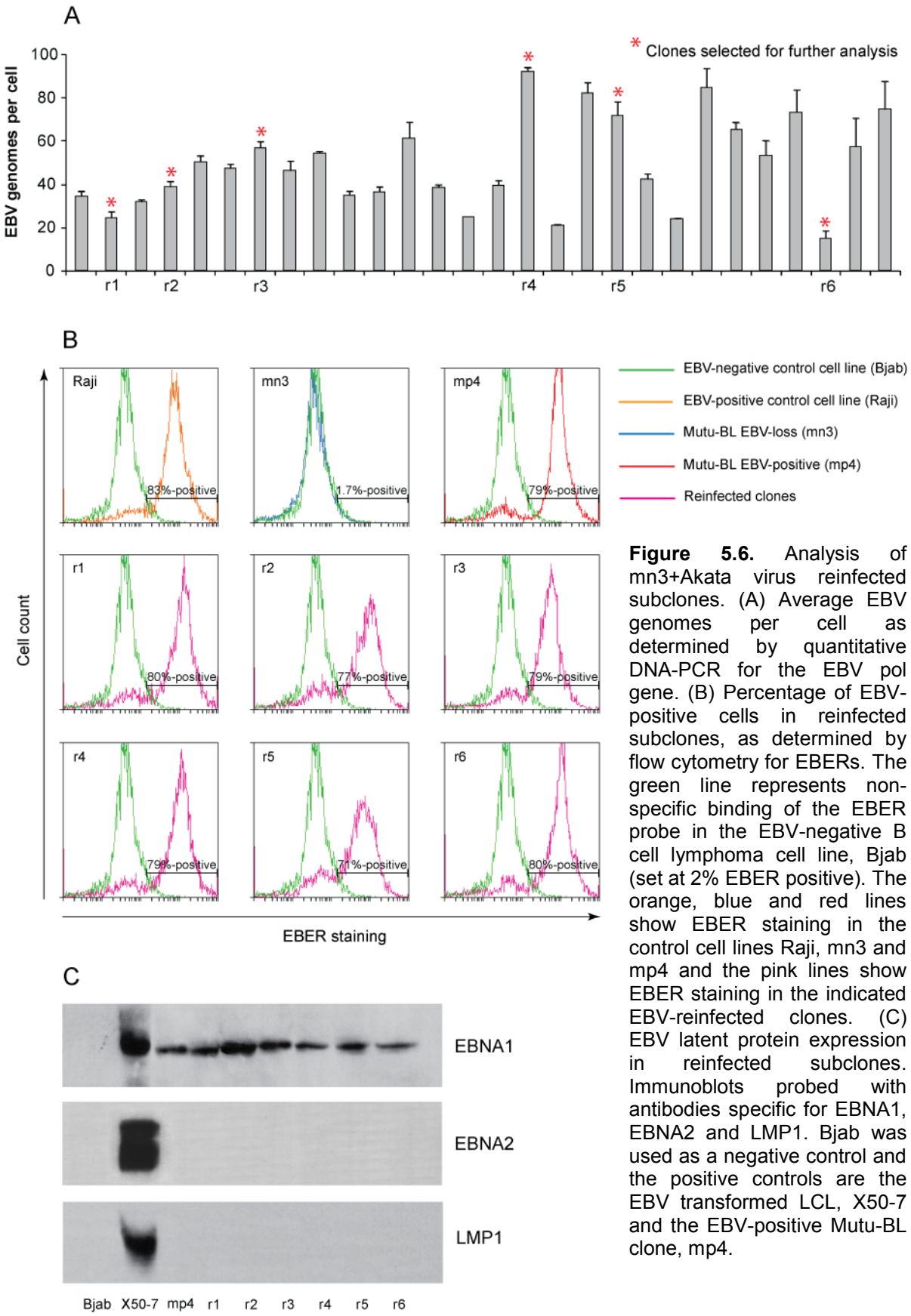


Figure 5.6. Analysis of mn3+Akata virus reinfected subclones. (A) Average EBV genomes per cell as determined by quantitative DNA-PCR for the EBV pol gene. (B) Percentage of EBV-positive cells in reinfected subclones, as determined by flow cytometry for EBERs. The green line represents non-specific binding of the EBER probe in the EBV-negative B cell lymphoma cell line, Bjab (set at 2% EBER positive). The orange, blue and red lines show EBER staining in the control cell lines Raji, mn3 and mp4 and the pink lines show EBER staining in the indicated EBV-reinfected clones. (C) EBV latent protein expression in reinfected subclones. Immunoblots probed with antibodies specific for EBNA1, EBNA2 and LMP1. Bjab was used as a negative control and the positive controls are the EBV transformed LCL, X50-7 and the EBV-positive Mutu-BL clone, mp4.

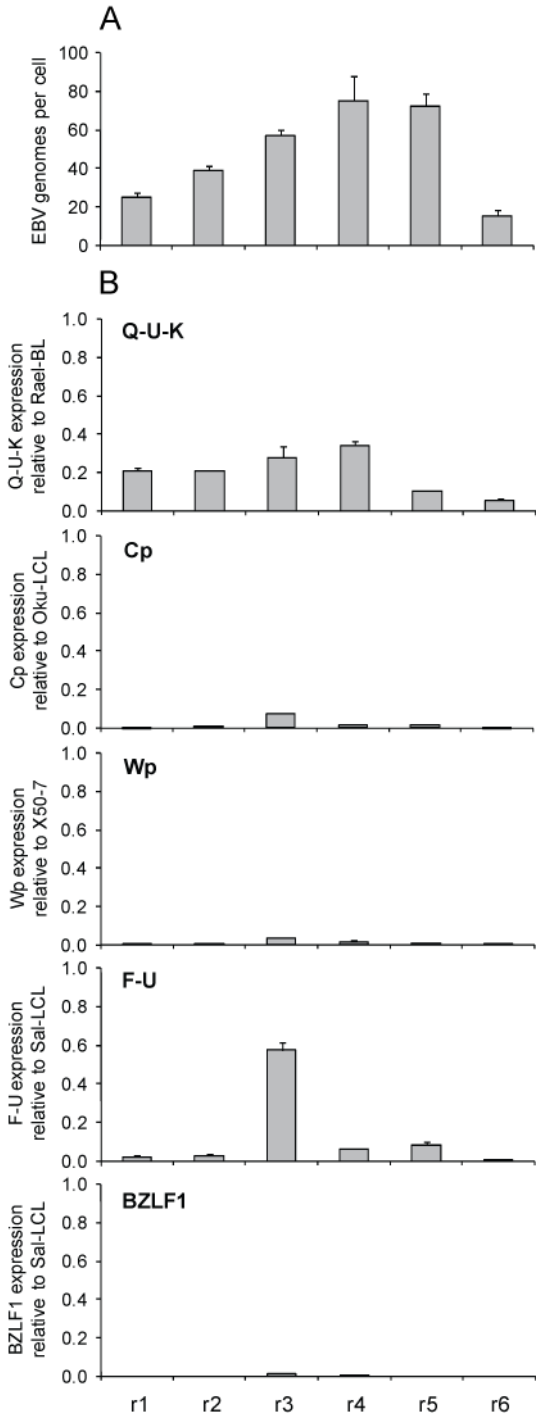


Figure 5.7. EBV latent gene expression in mn3+Akata virus reinfected subclones. (A) EBV genomes per cell as determined by quantitative DNA-PCR for the EBV pol gene. (B) EBV gene expression in EBV reinfected clones as determined by QRT-PCR. Transcription levels were measured relative to an appropriate control (assigned a value of 1) and error bars indicate the range between duplicate assays carried out in parallel on the same cDNA sample.

Next we investigated EBV viral latency by screening for viral protein expression by western blot (Figure 5.6(B)). All 6 clones expressed EBNA1 in the absence of EBNA2 or LMP1 so appeared to be conventional Latency I BL cells. Next we used specific QRT-PCR assays to investigate activity of the EBV latent promoters Qp, Cp and Wp and expression of the EBV lytic cycle transcripts, F-U and BZLF1 (Figure 5.7(A)). 3 clones (r1, r2 and r4) displayed typical Latency I viral gene expression by QRT-PCR. They expressed Qp derived Q-U-K transcripts (although at a slightly lower level than the Rael-BL standards) and showed little or no Cp or Wp activity. As observed in most EBV-positive BL clones, low level expression of lytic F-U transcripts could also be detected. This F-U expression was only a fraction of the level observed in the reference Sal-LCL which itself has only 2% of cells in lytic cycle and clones were almost completely negative for BZLF1. Thus it is highly unlikely that a significant number of cells were in active lytic cycle. In contrast, clone r3 showed higher Cp and Wp activity and expressed lytic F-U transcripts at nearly two thirds of the level observed in the lytic LCL control cell line. Clones r5 and r6 were broadly similar in gene expression to clones r1, r2 and r4; however they had reduced Qp activity. In conclusion it appears that by drug selection it is possible to restore Latency I EBV viral gene expression to EBV-loss clones. However, this is still a very rare event and the vast majority of infections result in the full Latency III growth transformation programme.

5.2.4 The effect of Latency I reinfection on apoptosis resistance

The isolation of Latency I reinfected cells allowed us to determine if restoration of EBV to EBV-loss clones restored resistance to apoptosis. Reinfected Mutu-BL clones r1, r2 and r4; EBV-positive Mutu-BL clones mp4 and mp5 and EBV-loss Mutu-BL clones mn1, mn2 and mn3 were treated for 48 hours with 0.5µg/mL ionomycin. Apoptosis and necrosis were then determined by Syto 16 and propidium iodide staining. The percentage induced apoptosis and necrosis is shown in Figure 5.8. In agreement with earlier data, ionomycin induced cell death occurred predominantly by apoptosis. In this experiment 0.5µg/ml ionomycin induced cell death in nearly 20% of cells from the EBV-positive clones, mp4 and mp5, but induced between 50-60% cell death in the EBV-loss clones, mn1, mn2 and mn3. Restoration of Latency I viral gene expression in reinfected clones r1, r2 and r4 reduced ionomycin induced cell death back to the level observed in EBV-positive clones. This confirms that at least in Mutu-BL, the apoptosis resistance observed in Latency I EBV-positive clones is virally mediated.

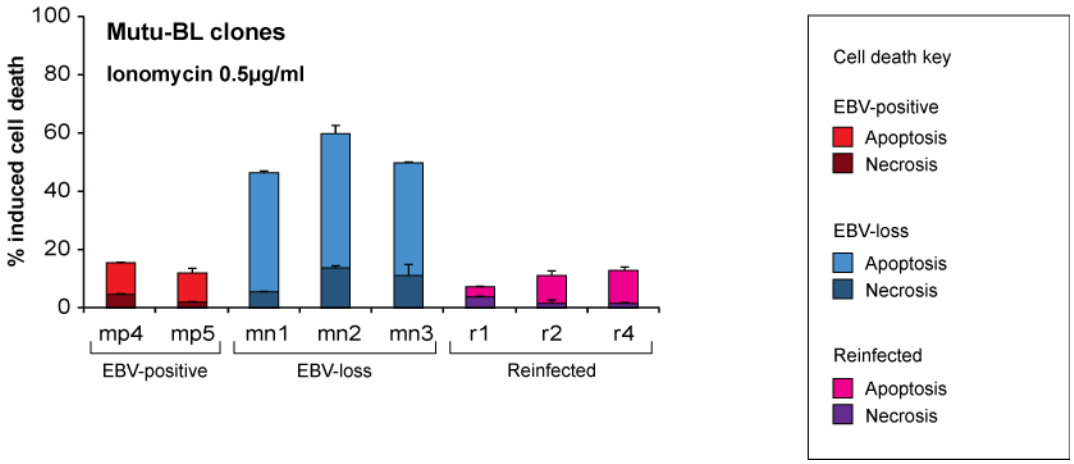


Figure 5.8. Apoptosis resistance in mn3+Akata virus reinfected subclones. Cell death in EBV-positive, EBV-loss and EBV reinfected clones of Mutu-BL after 48 hour treatment with ionomycin, as determined by Syto 16 and propidium iodide staining. The lighter coloured section of each bar represents induced apoptosis and the darker section is induced necrosis. Error bars represent standard deviation in cell death across 3 parallel wells from the same experiment.

5.3 The effect of Latency I gene product expression in EBV-loss clones

We have shown that loss of EBV from 5 different BL cell lines consistently sensitised cells to apoptosis, which in the case of Mutu-BL could be restored by reinfection with a recombinant virus. However, viral gene expression in these EBV-positive clones is limited to the viral genome maintenance protein EBNA1, the non-coding EBER transcripts, the BARTs, which may have a protein coding capacity and a growing collection of EBV encoded microRNAs. In addition, if lytically active cells are also present in the culture, vIL-10 may be released into the culture medium which could affect surrounding cells.

At the time of this investigation, the EBNA1 protein had been implicated in apoptosis resistance, possibly through modulation of the p53 pathway (Kennedy et al., 2003; Saridakis et al., 2005) and a protective role for the EBERs had also been shown through binding to the antiviral protein, PKR (Sharp et al., 1993; Nanbo and Takada, 2002) and by stimulation of IL-10 production (Kitagawa et al., 2000; Samanta et al., 2008). Thus we investigated if expression of EBNA1 or EBERs could restore resistance to apoptosis to EBV-loss clones.

5.3.1 Expression of EBNA1 in EBV-loss clones

We decided first to concentrate on EBNA1, since it is the only viral protein expressed in Latency I. To assess EBNA1's role in apoptosis resistance in BL cells, we used 2 episomally maintained plasmid vectors (pRTS-CD2 and pRTS EBNA1 CD2) originally developed by Georg Bornkamm and kindly offered for our use in this work. Simplified maps of pRTS-CD2 and pRTS EBNA1 CD2 are shown in Figure 5.9 and a detailed description of their construction can be found in section 2.15.1. Both pRTS vectors constitutively express rat CD2 from an SV40 promoter which allows vector positive populations to be enriched using magnetic activated cell sorting (MACS). They also constitutively express EBNA1 from a cryptic promoter and encode the EBV origin of replication (oriP). Together EBNA1 and oriP prevent loss of the pRTS plasmid vectors from cells by promoting their replication and segregation to daughter cells during cell division.

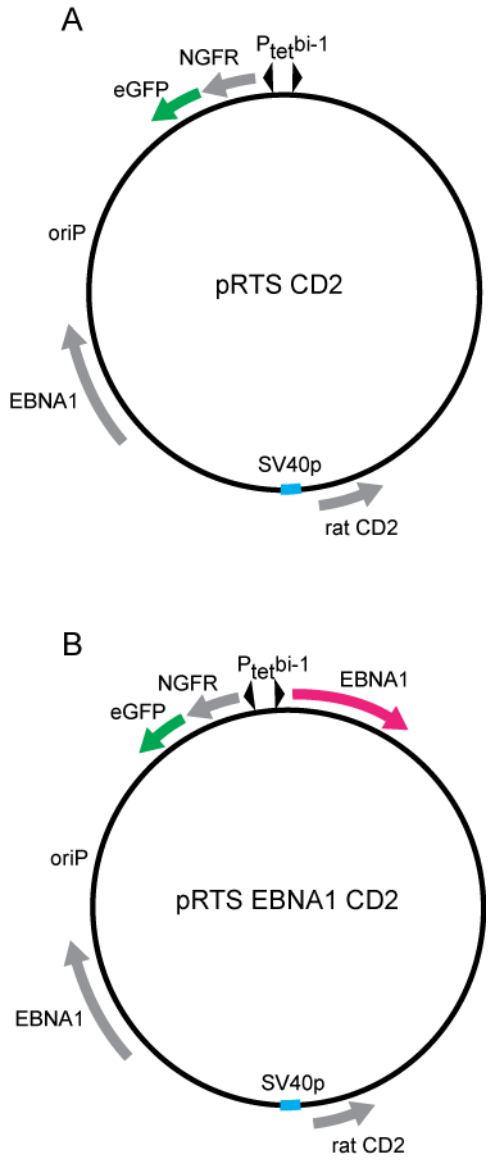


Figure 5.9. Simplified maps of pRTS CD2 vectors: (A) pRTS-CD2 and (B) pRTS EBNA1 CD2. P_{tet}bi-1 denotes the bidirectional tetracycline-regulated promoter, which drives expression of NGFR, eGFP and, in pRTS EBNA1 CD2 expression of the EBV genome maintenance protein, EBNA1. Expression of rat CD2 is driven by the SV40 promoter (SV40p) and constitutive EBNA1 expression in both vectors is driven by a cryptic promoter.

The ability to enrich pRTS vector transfected populations on the basis of rat CD2 and the episomal maintenance of plasmids by EBNA1 and oriP negates the need for drug selection to generate stable vector positive cell lines. As mentioned previously, methods which avoid antibiotic selection are preferable as drug selection can itself affect resistance to apoptosis. Both vectors contain the tetracycline/doxycycline bi-directional promoter, P_{tet}bi-1. In pRTS-CD2, activation of P_{tet}bi-1 by doxycycline (dox) induces expression of enhanced green fluorescent protein (eGFP) and the nerve growth factor receptor (NGFR) from a bicistronic mRNA. eGFP expression was used as a marker for pRTS vector transfected cells during flow cytometric analysis and can also be used as an alternative marker for sorting pRTS vector positive cells by fluorescence activated cell sorting (FACS). pRTS EBNA1 CD2 also has a second, inducible, copy of EBNA1 cloned adjacent to P_{tet}bi-1. Thus, in addition to eGFP and NGFR expression, activation of P_{tet}bi-1 in pRTS EBNA1 CD2 transfected cells also induces additional EBNA1 expression above the level due to constitutive EBNA1 expression from the vector backbone. In the following experiments, we determined the effects of EBNA1, either expressed at low levels from the pRTS backbone or over expressed from the inducible pRTS EBNA1 CD2 construct, on the apoptosis sensitivity of EBV-loss BL clones.

As outlined in Figure 5.10, different sorting protocols were used for the sorting of pRTS CD2 positive Akata-BL and Kem-BL clones. pRTS-CD2 and pRTS EBNA1 CD2 were transfected into EBV-loss clones of Akata-BL and Kem-BL by electroporation. Vector positive cells were enriched in EBV-loss clones of Akata-BL using MACS sorting as described in section 2.17.2. However, the more sensitive EBV-loss clones of Kem-BL could not tolerate the MACS sorting protocol, so transfected cells were induced with 50ng/mL dox for 48 hours and sorted by FACS for eGFP expression (section 2.17.1). Both sorting protocols yielded only a small number of cells, so cell populations were expanded before being induced with dox and investigated for EBNA1 expression and resistance to apoptosis. To account for any changes which may have occurred in Kem-BL cells due to the cell sorting procedure GFP-negative cells were also simultaneously collected from the sort and used as a control in the apoptosis assays.

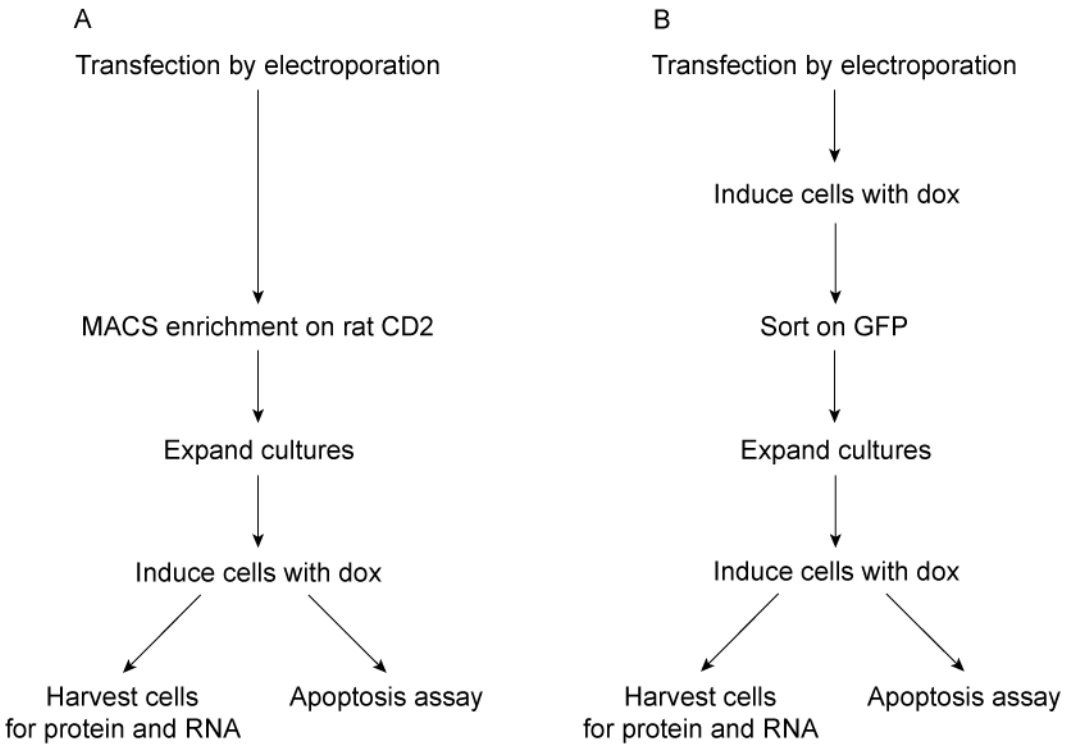


Figure 5.10. Strategy used to deliver pRTS vectors to EBV-loss clones of (A) Akata-BL, (B) Kem-BL.

We first confirmed constitutive and inducible EBNA1 expression in sorted populations of pRTS vector positive cells by western blotting and immunofluorescence (IF). EBNA1 expression in sorted Akata-BL and Kem-BL cells is shown in Figure 5.11 and Figure 5.12 respectively. Akata-BL cells transfected with pRTS-CD2 constitutively express EBNA1 at similar levels to the EBV-positive Akata-BL clones, ap2 and ap3. Cells transfected with pRTS EBNA1 CD2 expressed around twice the level of EBNA1 seen in EBV-positive clones when grown in normal BL medium. This increased to around 3 times normal EBNA1 expression after the addition of 50ng/ml dox and to around 4 times normal EBNA1 expression after the addition of 1000ng/ml dox. Note that Dox induced EBNA1 had a slightly lower molecular weight than EBNA1 expressed from the pRTS-CD2 vector backbone or in EBV-positive BL cell lines, which may result from a slight reduction in the number of Gly-Ala repeats in this second copy of EBNA1. Strong EBNA1 staining could be seen by IF in most pRTS transfected cells indicating that the physiological levels of EBNA1 expression seen by immunoblotting were not a result of high EBNA1 expression from only a small fraction of the transfected cells, but rather physiological EBNA1 expression in the majority of cells.

EBV-loss clones of Kem-BL transfected with pRTS CD2 expressed levels of EBNA1 comparable with EBV-positive Kem-BL clones, kp1 and kp2. Cells transfected with pRTS EBNA1 CD2 also expressed similar levels of EBNA1 to EBV-positive Kem-BL clones when grown in normal medium. As observed in clones of Akata-BL, addition of dox to pRTS EBNA1 CD2 transfected cells induced a dose-dependent increase in EBNA1 expression. Incubation with 50ng/ml dox increased EBNA1 expression by around 2-fold, while 1000ng/ml dox increased EBNA1 expression to roughly 4 times the level observed in EBV-positive Kem-BL clones. As was seen in the Akata-BL background, EBNA1 induced by the addition of dox had a slightly lower molecular weight than EBNA1 expressed from the vector backbone.

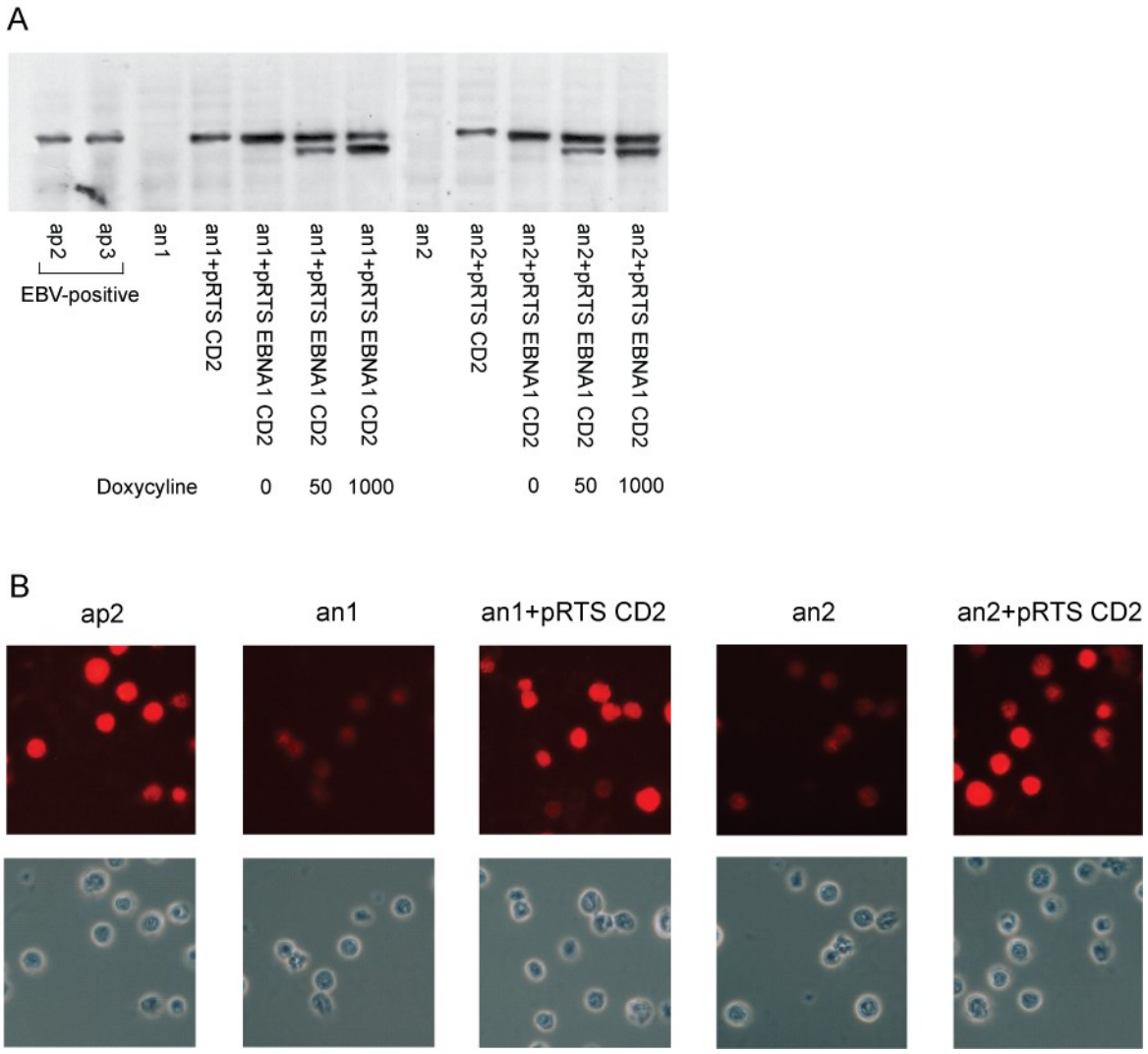


Figure 5.11. EBNA1 expression in EBV-loss Akata-BL clones transfected with pRTS vectors. (A) Immunoblot of EBNA1 expression in pRTS CD2 and pRTS EBNA1 CD2 transfected cells. To demonstrate dox mediated induction of EBNA1 in pRTS EBNA1 CD2 transfected cells, cells were incubated in normal medium or medium containing 50ng/ml or 1000ng/ml doxycycline. The positive controls were the two EBV-positive Akata-BL clones, ap2 and ap3 and the negative controls were the untransfected EBV-loss clones, an1 and an2. (B) IF staining for EBNA1 in cells transfected with pRTS CD2 with a bright field image of the same area shown below.

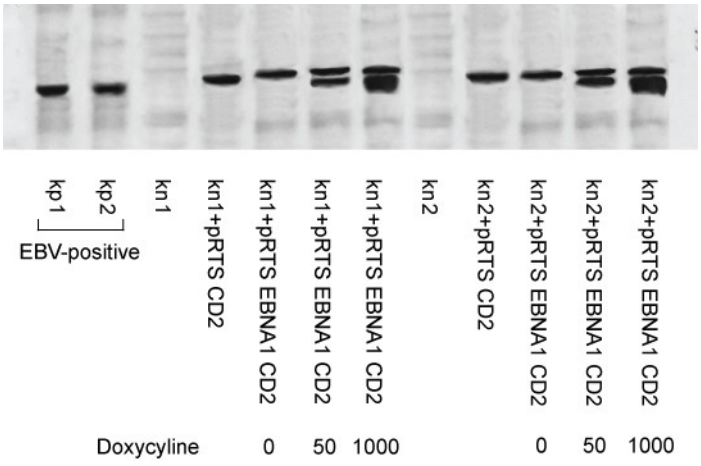


Figure 5.12. EBNA1 expression in EBV-loss Kem-BL clones transfected with pRTS vectors. EBNA1 expression determined by immunoblotting with an EBNA1 specific antibody in pRTS CD2 and pRTS EBNA1 CD2 transfected cells. To demonstrate dox mediated induction of EBNA1 in pRTS EBNA1 CD2 transfected cells, cells were incubated in normal medium or medium containing 50ng/ml or 1000ng/ml doxycycline. The positive controls were the two EBV-positive Kem-BL clones, kp1 and kp2 and the negative controls were the untransfected EBV-loss clones, kn1 and kn2.

5.3.2 Effect of EBNA1 on apoptosis resistance in EBV-loss clones of Akata-BL

To determine if expression of EBNA1 from either pRTS CD2 or pRTS EBNA1 CD2 could restore resistance to apoptosis in EBV-loss clones, we used a slightly modified version of the standard apoptosis assay. $1.5x10^4$ EBV-positive, EBV-loss and pRTS vector transfected cells were transferred to wells of a 96 well plate in 200µL of normal BL medium or medium supplemented with 50ng/mL or 1000ng/mL dox. After 24 hours, 100µL of medium was removed and replaced with 100µL of BL medium containing the appropriate dox concentration plus 2x concentration of apoptosis inducing agent (ionomycin, anti-IgM or roscovitine). As a negative control, the medium from several wells was also replaced with normal BL medium containing only the appropriate dox concentration. Apoptosis was induced for 48 or 72 hours (depending on the apoptosis inducing agent) and cells were harvested and stained with propidium iodide (PI) as described in section 2.5.1. Syto 16 staining could not be used as it is detected by the same colour channel as GFP. By altering the compensation setting of the flow cytometric analysis it was possible to use PI staining alone to examine total cell death in each sample. This avoided the interference between GFP and Syto 16 signals, but meant that this assay was not able to discriminate between apoptosis and necrosis. Instead total cell death was determined by plotting a 2 dimensional dot plot of PI staining against GFP expression (Figure 5.13(A)).

pRTS vector transfected cells sorted on rat CD2 or GFP were around 95% vector positive immediately after sorting. However despite the presence of EBNA1 and oriP, the number of vector positive cells slowly decreased over time. At the point at which cells were analysed for apoptosis resistance, the percentage of pRTS positive cells varied from 40-80%. To account for this, percentage induced cell death in transfected cells was calculated as the percentage of GFP-positive cells that were induced to die. Indeed the presence of pRTS vector negative cells in the culture provided a useful internal control since the percentage induced cell death of GFP-positive/vector-positive cells could be directly compared to GFP-negative/vector-negative cells in the same population.

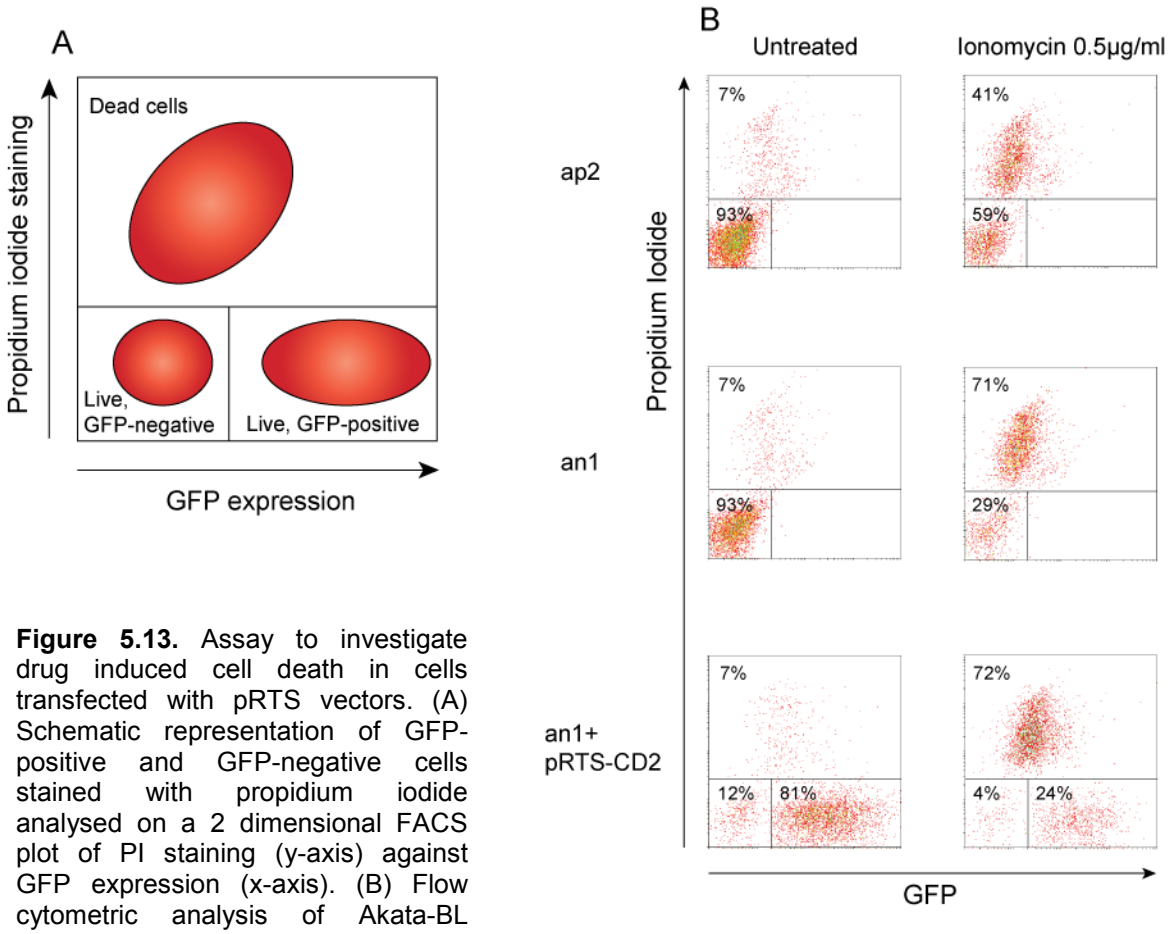


Figure 5.13. Assay to investigate drug induced cell death in cells transfected with pRTS vectors. (A) Schematic representation of GFP-positive and GFP-negative cells stained with propidium iodide analysed on a 2 dimensional FACS plot of PI staining (y-axis) against GFP expression (x-axis). (B) Flow cytometric analysis of Akata-BL clones, ap2, an1 and an1+ pRTS-CD2 after treatment with ionomycin and staining with propidium iodide.

The top and middle panels of Figure 5.13(B) show flow cytometric data from a single EBV-positive clone (ap2) and EBV-loss clone (an1) of Akata-BL. The bottom panel shows data from the same EBV-loss clone (an1) after transfection with pRTS CD2 and activation with dox. After PI staining, flow cytometry was used to construct a 2 dimensional dot plot of PI staining (y-axis) versus GFP expression (x-axis). Ionomycin induced cell death is clearly detected as an increase in PI uptake in all 3 cultures using this alternative flow cytometric program. GFP expression can only be detected in live cells so it was not possible to differentiate between dead GFP-positive and dead GFP-negative cells. Thus, induced cell death was calculated from the reduction in the percentage of cells in the live GFP-positive and live GFP-negative populations. If we use the values from the pRTS CD2 transfected culture from Figure 5.13 as an example, the untreated cell population was found to be 7% dead cells, 12% live, GFP-negative and 81% live, GFP-positive. After treatment with 0.5µg/ml ionomycin, the percentage GFP-negative, live cells decreases to 4%, thus the percentage induced cell death in the live, GFP-negative population is ((12 – 4) ÷ 12) x 100 = 66.7%. Similarly the percentage live GFP-positive cells decreases to 24%; thus the induced cell death in the live GFP-positive population is ((81 – 24) ÷ 81) x 100 = 70.4%.

Figure 5.14 shows flow cytometric cell death data summarised into bar charts from 2 EBV-positive Akata-BL clones (ap2 and ap3), 2 Akata-BL EBV-loss clones (an1 and an2) and an1 and an2 after transfection with either pRTS-CD2 or pRTS EBNA1 CD2. To investigate whether EBNA1 could restore resistance to apoptosis, Akata-BL clones were treated with 0.5µg ionomycin or 50µM roscovitine. This dose of ionomycin induced around 40% cell death in EBV-positive clones of Akata-BL and 70-80% cell death in the EBV-loss clones. Constitutive expression of physiological levels of EBNA1 from the backbone pRTS-CD2 vector in EBV-loss clones appeared to have no effect on resistance to apoptosis; cell death was equivalent in pRTS-CD2 transfected cells and EBV-loss cells. As a control, dox was titrated into the medium, leading to expression of eGFP and NGFR from the bi-directional tetracycline/doxycycline responsive promoter (P_{tet}bi-1) and this in fact made cells slightly more sensitive to apoptosis. This is likely to be due to the low level toxicity of dox rather than because of eGFP or NGFR expression.

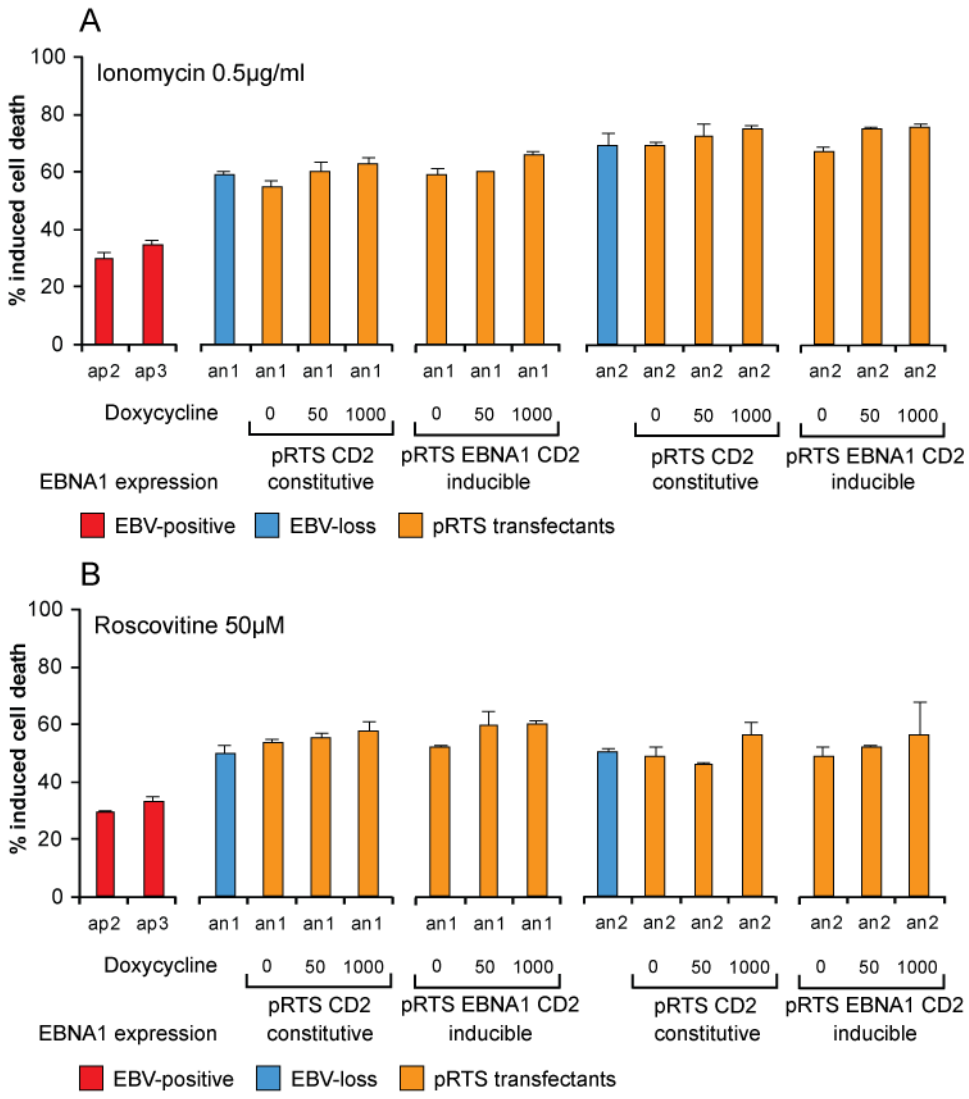


Figure 5.14. Effect of EBNA1 expression on resistance of clones of Akata-BL to apoptosis induced by (A) 0.5µg/ml ionomycin or (B) 50µM roscovitine. Cell death in cells induced with dox was calculated as the percentage of GFP-positive cells induced to die. For those cells not induced with dox total cell death was recorded. Error bars represent standard deviation in cell death across 3 parallel wells from the same experiment.

In Figure 5.14, the induced cell death shown for dox induced cultures is the percentage of GFP-positive cells induced to die; however the percentage cell death in GFP-negative cells was also examined and was found to be very similar to the GFP-positive cells. This provides another indication that EBNA1 is not affecting apoptosis sensitivity.

To further show that EBNA1 could not protect cells from apoptosis, we decided to overexpress EBNA1 in EBV-loss Akata-BL cells by using the pRTS EBNA1 CD2 vector where EBNA1, in addition to being constitutively active in the backbone, had been cloned downstream of P_{tet}bi-1. In the absence of dox, induced cell death in pRTS EBNA1 CD2 transfected cells was equal to cell death in untransfected EBV-loss clones. Overexpression of EBNA1 from P_{tet}bi-1 via the addition of 50ng/mL or 1000ng/mL dox also gave no protection from apoptosis. In fact cells were slightly more sensitive to death induction, probably due to the addition of dox.

Investigation of resistance to roscovitine yielded very similar results to those observed after ionomycin treatment. 50μM roscovitine induced between 20-30% cell death in EBV-positive clones of Akata-BL and approximately 50% cell death in EBV-loss clones. Transfection of pRTS-CD2 or pRTS EBNA1 CD2 had no effect on resistance to roscovitine induced cell death. Cell death in transfected populations in the absence of dox was equal to the induced cell death in untransfected EBV-loss clones. Activation of pRTS-CD2 and pRTS EBNA1 CD2 again did not protect cells from apoptosis.

5.3.3 Effect of EBNA1 on apoptosis resistance in EBV-loss clones of Kem-BL

To confirm the effect of EBNA1 expression observed in Akata-BL clones we used pRTS CD2 and pRTS EBNA1 CD2 to express EBNA1 in Kem-BL cells. To determine the effect on apoptosis, we used the same modified apoptosis assay as used for transfected clones of Akata-BL; however, because Kem-BL clones were susceptible to anti-IgM cross-linking, we replaced roscovitine with anti-IgM as a second apoptosis inducing agent. Figure 5.15 shows flow cytometric cell death data summarised into bar charts from 2 EBV-positive Kem-BL clones (kp1 and kp2), 2 Kem-BL EBV-loss clones (kn1 and kn2) and kn1 and kn2 after transfection with either pRTS-CD2 or pRTS EBNA1 CD2. To induce sufficient levels of apoptosis in Kem-BL clones we used 0.5μg ionomycin or 5μg/ml Anti-IgM cross-linking antibody. This dose of ionomycin induced around 20% cell death in EBV-positive clones of Kem-BL and 50% cell death in the EBV-loss clones. Constitutive expression of physiological levels of

EBNA1 from the backbone pRTS-CD2 vector in EBV-loss clones again appeared to have no effect on resistance to apoptosis; as observed in clones of Akata-BL, cell death was equivalent in EBV-loss and pRTS-CD2 transfected cells. Activation of the P_{tet}bi-1 promoter with dox again appeared to cause a slight increase in susceptibility of cells to apoptosis. Once again there was no difference between cell death in the GFP-positive and GFP-negative dox-induced populations. As a further control, we also carried out the apoptosis assay on GFP-negative cells collected during the FACS protocol used to isolate pure transfected populations. We found that there was no difference between the sensitivity of these GFP-negative cells and the GFP-positive cells which carried the expression vectors (data not shown), indicating once again that EBNA1 is unable to modulate resistance to apoptosis and demonstrating that the sensitivity of Kem-BL cells is not affected by the sorting protocol. As observed in Akata-BL cells, overexpression of EBNA1 in pRTS EBNA1 CD2 transfected cells, by the addition of dox, was also unable to prevent ionomycin induced apoptosis.

Investigation of resistance to anti-IgM cross linking yielded very similar results to those observed after ionomycin treatment. 5µg/ml anti-IgM induced 40% cell death in EBV-positive clones of Kem-BL and approximately 80% cell death in EBV-loss clones. Transfection of pRTS-CD2 or pRTS EBNA1 CD2 had no effect on resistance to anti-IgM induced cell death. Cell death in transfected populations in the absence of dox was equal to the induced cell death in untransfected EBV-loss clones. Activation of pRTS-CD2 and pRTS EBNA1 CD2 again did not protect cells from apoptosis.

As it appeared that expression of EBNA1 alone (even at levels several times higher than observed in EBV-positive clones) was unable to restore resistance to apoptosis in clones of Akata-BL or Kem-BL, we decided to determine the effect of dual expression of the EBNA1 protein and the non-coding EBER transcripts.

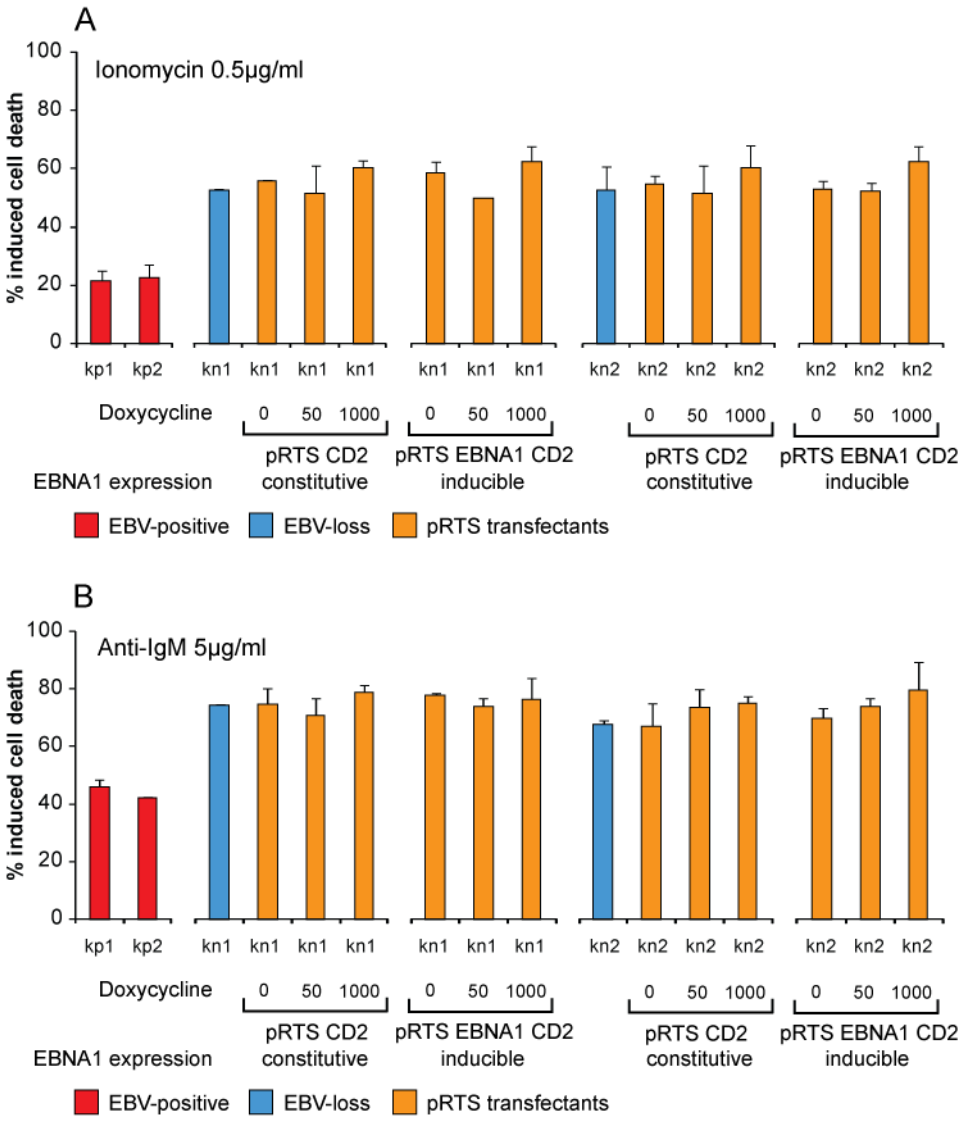


Figure 5.15. Effect of EBNA1 expression on resistance of clones of Kem-BL to apoptosis induced by (A) 0.5µg/ml ionomycin or (B) 5µg/ml Anti-IgM. Cell death in cells induced with dox was calculated as the percentage of GFP-positive cells induced to die. For those cells not induced with dox total cell death was recorded. Error bars represent standard deviation in cell death across 3 parallel wells from the same experiment.

5.3.4 Generation of a conditional EBER expression plasmid

To investigate the effect of EBER expression, we generated an EBNA1/EBER expression vector based on the pRTS-CD2 system developed by Georg Bornkamm (Bornkamm et al., 2005), which we named pRTS EBER CD2. The method used to generate pRTS EBER CD2 is described in detail in section 2.15.2. Briefly, conventional DNA-PCR was used to amplify a section of B95-8 DNA containing the EBER1 and EBER2 genes along with their respective promoters. This EBER fragment was then ligated into the intermediate cloning vector, pUC19 SfiI. The EBER fragment was excised from this intermediate cloning vector and cloned into the pRTS-CD2 backbone adjacent to the bi-directional tetracycline/doxycycline inducible promoter, P_{tet}bi-1. A simplified map of the generated pRTS EBER CD2 plasmid is shown in Figure 5.16 and validation of EBER1 and EBER2 expression from this newly generated plasmid is shown below.

Previous vectors used to express EBERs in BL cells have relied on multiple copies of the EBERs to induce physiological EBER expression. Using this episomally replicating dox inducible EBER expression system we aimed to investigate the effect of different levels of EBER expression using only a single copy of the EBER cassette without the need for drug selection to generate stable vector positive populations.

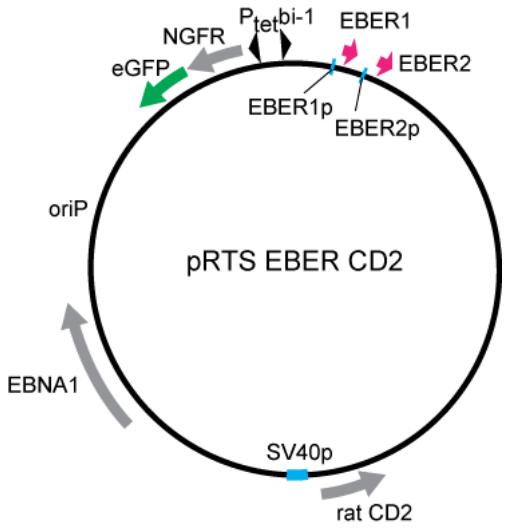


Figure 5.16. Simplified map of the pRTS EBER CD2 plasmid vector. P_{tet}bi-1 denotes the bidirectional tetracycline-regulated promoter, which upon addition of doxycycline to the cell medium drives expression of NGFR and eGFP in one direction and EBER1 and EBER2 in the opposite direction. Expression of rat CD2 is driven by the SV40 promoter (SV40p). OriP represents the EBV origin of replication and constitutive expression of the genome maintenance protein EBNA1 is driven by a cryptic promoter.

5.3.5 Regulatable expression of EBERs in EBV-loss clones

The pRTS EBER CD2 vector was transfected into 2 EBV-loss clones of Akata-BL and 2 EBV-loss clones of Kem-BL by electroporation and stable cell lines generated as previously described for pRTS-CD2 and pRTS EBNA1 CD2 vectors. To examine expression of the EBERs, cells were treated with a range of dox concentrations ranging from 5-1000ng/ml and after 48 hours RNA was extracted, DNase I treated to remove any possible contamination with plasmid DNA and reverse transcribed to cDNA using random primers. The expression level of EBER1 and EBER2 was determined using QRT-PCR and cells were also harvested to investigate constitutive EBNA1 expression from the vector backbone. Expression of EBERs from pRTS EBER CD2 in EBV-loss clones of Akata-BL and Kem-BL is shown in Figure 5.17 and Figure 5.18. For reference, Figure 5.17(A) shows expression of EBER1 and EBER2 relative to the EBV transformed LCL, X50-7 in EBV-positive and EBV-loss clones of Akata-BL. Figure 5.17(B) shows EBER1 and EBER2 expression in EBV-loss clones of Akata-BL transfected with pRTS EBER CD2. To compensate for the fact that not every cell within the transfected population was vector positive, the values in Figure 5.17(B) have been normalised to the percentage of GFP-positive cells as determined by flow cytometry and therefore show the EBER expression that would be observed if transfected populations were 100% pRTS EBER CD2 positive. We found that in the absence of dox, pRTS EBER CD2 transfected cells produced a small amount of EBER1 and EBER2 in EBV-loss clones of Akata-BL; this is likely to be due to expression of EBERs from their natural promoters. Addition of dox induced a dose-dependent increase in EBER expression up to a maximum of around 60x EBER1 expression and 100x EBER2 expression compared to X50-7. Note that while EBV-positive Akata-BL clones produce around 8-fold more EBER1 than EBER2 when compared to X50-7, dox-induced pRTS EBER CD2 transfected EBV-loss clones expressed roughly equal amounts of EBER1 and EBER2. The discrepancies in relative levels of EBER1 and EBER2 could be due to high EBER1 expression in Akata-BL clones or low EBER1 expression in the X50-7 control cell line.

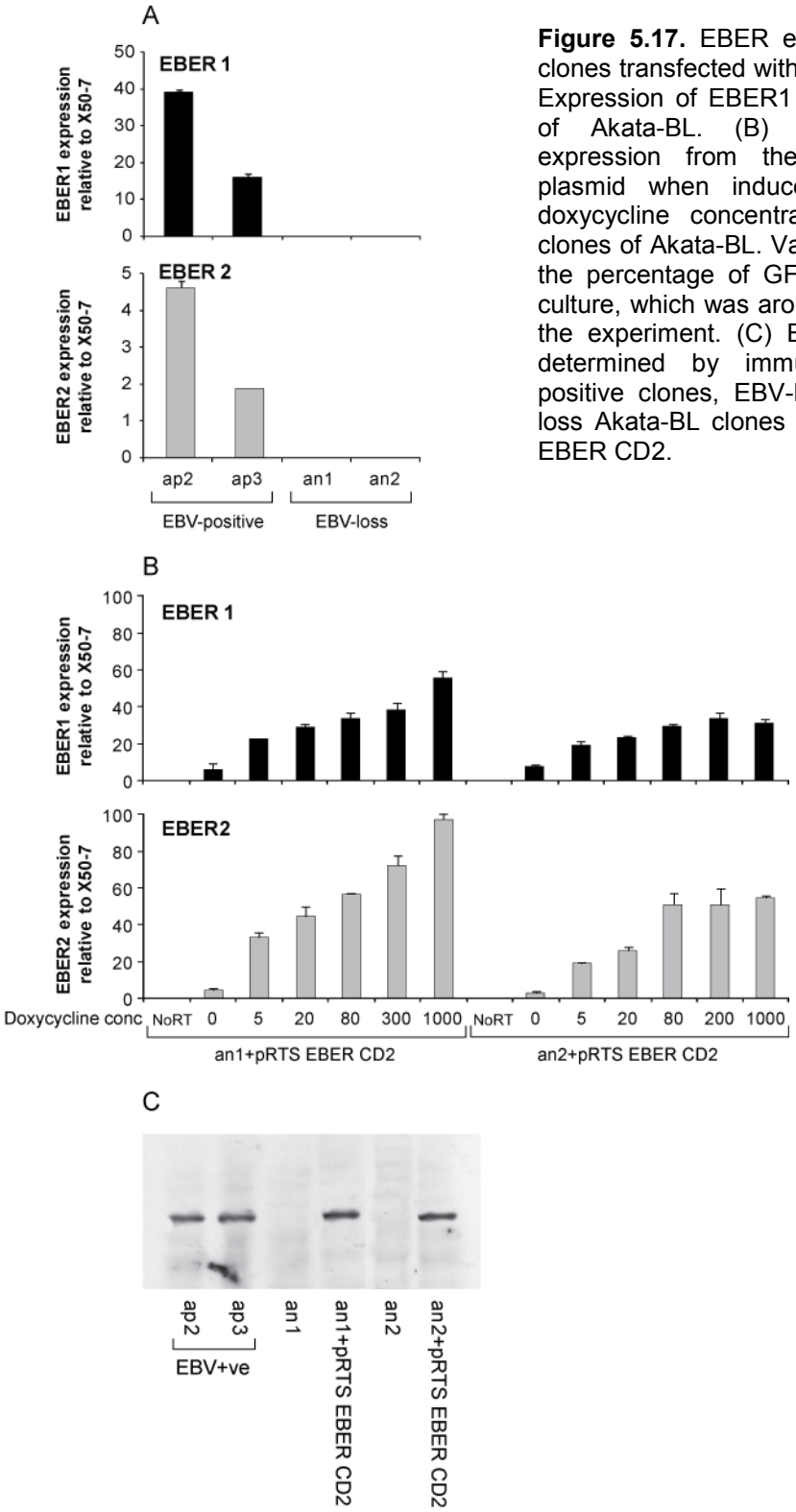


Figure 5.17. EBER expression in Akata-BL clones transfected with pRTS EBER CD2. (A) Expression of EBER1 and EBER2 in clones of Akata-BL. (B) Induction of EBER expression from the pRTS EBER CD2 plasmid when induced with a range of doxycycline concentrations in 2 EBV-loss clones of Akata-BL. Values are normalised to the percentage of GFP-positive cells in the culture, which was around 50% at the time of the experiment. (C) EBNA1 expression, as determined by immunoblotting, in EBV-positive clones, EBV-loss clones and EBV-loss Akata-BL clones transfected with pRTS EBER CD2.

We then performed the same investigation into EBER expression in EBV-loss clones of Kem-BL transfected with pRTS EBER CD2. Figure 5.18(A) shows expression of EBER1 and EBER2 relative to X50-7 in EBV-positive and EBV-loss clones of Kem-BL. Figure 5.18(B) shows EBER1 and EBER2 expression in EBV-loss clones of Kem-BL transfected with pRTS EBER CD2 normalised to the percentage of GFP-positive cells. Once again we found very low, but detectable levels of EBER1 and EBER2 in the absence of dox and a dose-dependent increase in EBER expression upon the addition of dox up to a maximum of 10x EBER1 and 35x EBER2 expression compared to the level observed in X50-7. Interestingly, although this pRTS EBER CD2 driven EBER expression is lower than the level observed in Akata-BL cells, it still correlates with the EBER expression observed in the EBV-positive Kem-BL clones (Figure 5.18(A)). Thus we again decided to use 50ng/mL dox to induce roughly physiological levels of EBERs and 1000ng/mL dox to overexpress EBERs. As shown in Figure 5.18 (C), EBV-loss Kem-BL clones transfected with pRTS EBER CD2 also expressed levels of EBNA1 similar to those observed in EBV-positive clones, kp1 and kp2.

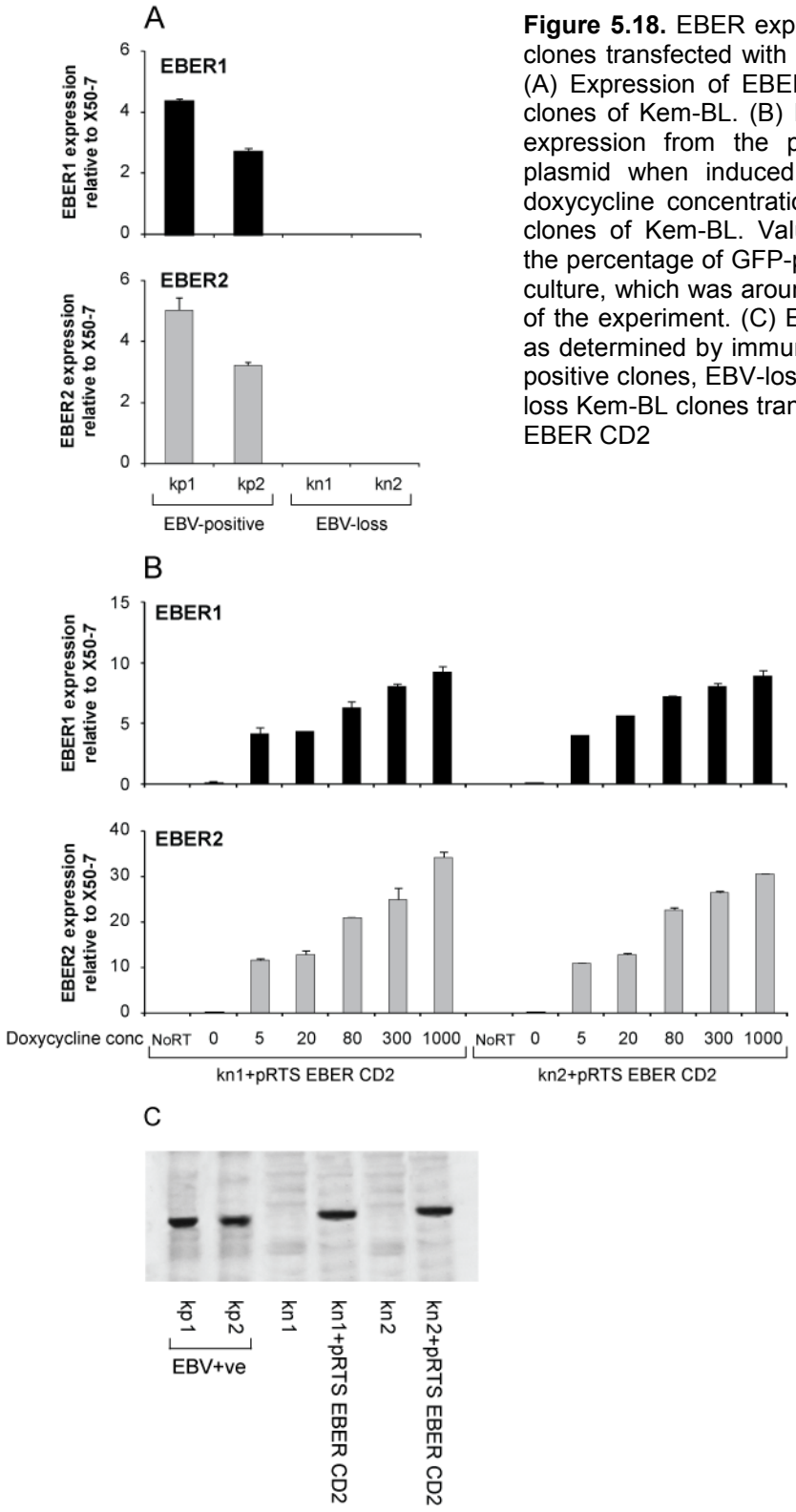


Figure 5.18. EBER expression in Kem-BL clones transfected with pRTS EBER CD2. (A) Expression of EBER1 and EBER2 in clones of Kem-BL. (B) Induction of EBER expression from the pRTS EBER CD2 plasmid when induced with a range of doxycycline concentrations in 2 EBV-loss clones of Kem-BL. Values normalised to the percentage of GFP-positive cells in the culture, which was around 20% at the time of the experiment. (C) EBNA1 expression, as determined by immunoblotting, in EBV-positive clones, EBV-loss clones and EBV-loss Kem-BL clones transfected with pRTS EBER CD2

5.3.6 Effect of EBERs on apoptosis resistance in EBV-loss clones of Akata-BL

To investigate if co-expression of EBNA1 and EBERs could restore apoptosis resistance in Akata-BL cells, 2 EBV-loss clones of Akata-BL were transfected with pRTS EBER CD2 or with pRTS-CD2 as a control and stable cell lines generated as described for pRTS-CD2 and pRTS EBNA1 CD2. Aliquots of cells were transferred to fresh BL medium or medium containing 50ng/mL or 1000ng/mL dox. After 48 hours, cells were harvested for RNA extraction and EBER1 and EBER2 expression were analysed by QRT-PCR. In parallel, apoptosis resistance was simultaneously measured in matching aliquots of pRTS-CD2 and pRTS EBER CD2 transfected cells using the modified version of the apoptosis assay previously described in section 5.3.2. Briefly, EBV-positive Akata-BL clones (ap2 and ap3), EBV-loss Akata-BL clones (an1 and an2) and an1 and an2 transfected with pRTS-CD2 or pRTS EBER CD2 were induced into apoptosis for 48 hours with 0.5µg/ml ionomycin or 50µM roscovitine. To determine the effect of EBER induction on ionomycin and roscovitine induced cell death, transfected cells were also examined after treatment with 50ng/ml or 1000ng/ml dox. Cell death was determined by staining with PI and flow cytometric analysis of PI staining against GFP expression; in dox induced cells, cell death was recorded as the percentage of GFP positive cells induced to die.

Figure 5.19 shows the effect of EBNA1 and EBER expression on resistance of Akata-BL clones to apoptosis inducing agents, ionomycin and roscovitine. Figure 5.19(A) shows EBER1 and EBER2 expression in EBV-positive Akata-BL clones (ap2 and ap3), EBV-loss Akata-BL clones (an1 and an2) and an1 and an2 transfected with pRTS-CD2 or pRTS EBER CD2. As expected there was no EBER expression in EBV-loss clones or EBV-loss clones transfected with pRTS-CD2. In the absence of dox, EBER1 and EBER2 expression in an1 and an2 transfected with pRTS EBER CD2 was roughly 1 twentieth of the level observed in EBV-positive clones (ap2 and ap3). Addition of 50ng/mL dox caused an increase in EBER1 expression to roughly the level observed in ap2 and ap3 and an increase in EBER2 expression to approximately 4 times ap2 and ap3 levels. Addition of 1000ng/ml dox increased EBER1 and EBER2 expression a further 2-fold compared to the level observed after treatment with 50ng/ml dox. As previously described, EBER expression was normalised to the percentage of GFP-positive cells in transfected populations as determined by flow cytometry (around 50% in this experiment).

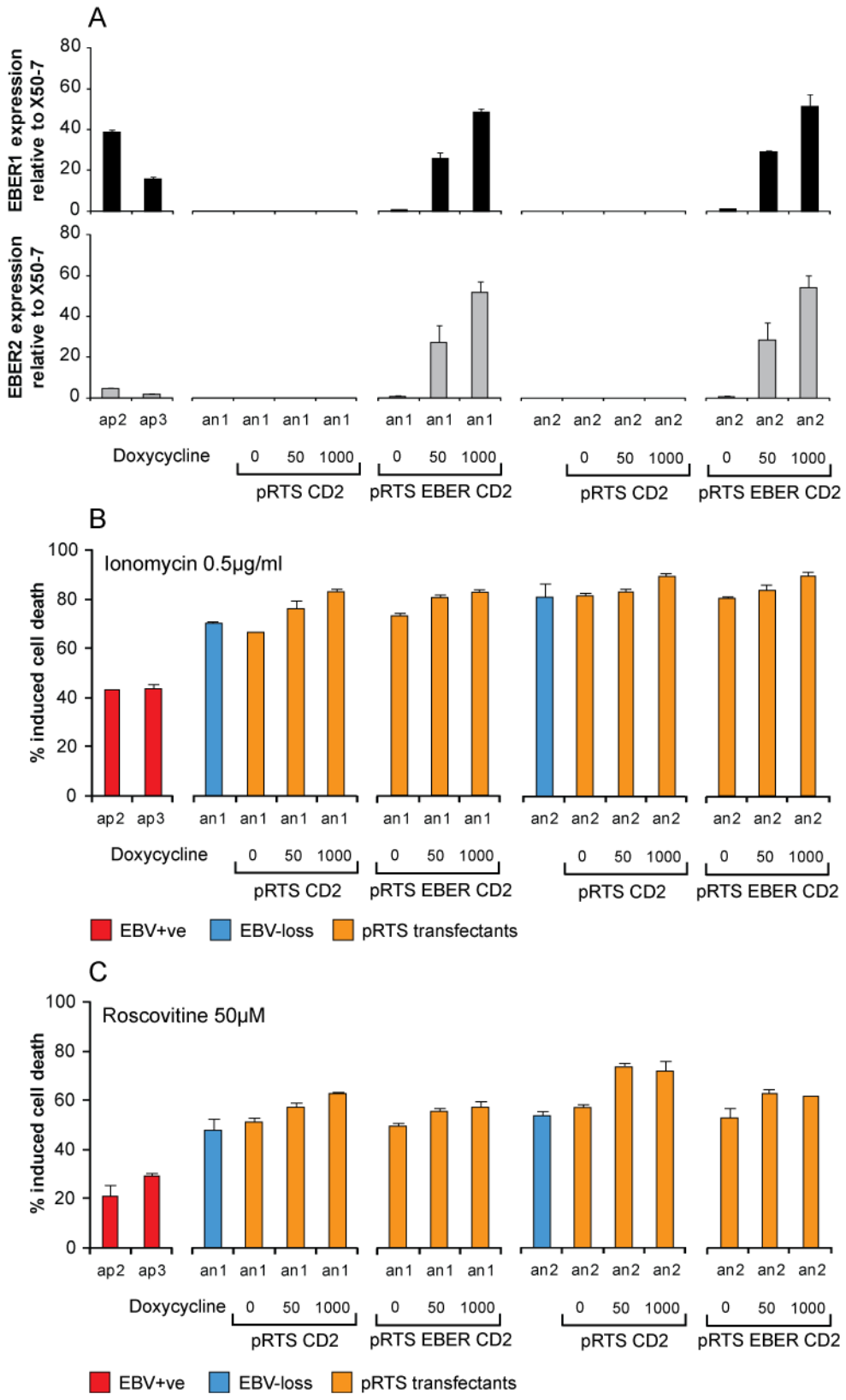


Figure 5.19. Effect of EBNA1 and EBERs on resistance to apoptosis in clones of Akata-BL. (A) Expression of EBER1 and EBER2 in EBV-positive Akata-BL clones, EBV-loss Akata-BL clones and EBV-loss Akata-BL clones transfected with pRTS CD2 and pRTS EBER CD2. EBER expression was determined by QRT-PCR and measured relative to the X50-7 LCL. (B and C) Effect of EBNA1 and EBERs on resistance of clones of Akata-BL to cell death induced by 0.5µg/ml ionomycin or 50µM roscovitine. Cell death in cells induced with dox was calculated as the percentage of GFP-positive cells induced to die. For those cells not induced with dox total cell death was recorded. Error bars represent standard deviation in cell death across 3 parallel wells from the same experiment.

Figure 5.19(B) compares the effect on apoptosis of co-expression of EBERs and EBNA1 against EBNA1 expression alone. As previously demonstrated, loss of EBV from Akata-BL cells increased the percentage of cells induced to die by 0.5µg/mL ionomycin from 40% in EBV-positive clones to 70-80% in EBV-loss clones. This increased sensitivity of EBV-loss clones to ionomycin was not reversed by the expression of EBNA1 from pRTS-CD2 (Figure 5.14). Furthermore we also found that induced cell death was also unaffected by expression of the EBNA1 plus the EBERs. Cell death in pRTS EBER CD2 transfected cells in the absence of dox was very similar to the level observed in EBV-loss clones, despite the low level EBER expression. Induction of EBER expression to the levels observed in EBV-positive Akata-BL cells with 50ng/mL dox or to approximately twice the level observed in Akata-BL with 1000ng/mL dox was also unable to rescue cells from ionomycin induced cell death. As observed during investigation of the effect of EBNA1 on resistance to cell death, we found that the addition of dox actually caused a small dose-dependent increase in cell death in pRTS EBER CD2 transfected cells. We found very similar results when we investigated the effect of EBER expression on resistance to roscovitine induced cell death. Loss of EBV from Akata-BL clones sensitised cells to roscovitine induced cell death. However, expression of EBNA1 alone (from pRTS-CD2) or EBNA1 and EBERs (from pRTS EBER CD2) was unable to reverse this apoptosis sensitivity.

5.3.7 Effect of EBERs on apoptosis resistance in EBV-loss clones of Kem-BL

To confirm the effect of EBNA1 and EBER expression observed in Akata-BL clones we used pRTS EBER CD2 to co-express EBNA1 and EBERs in Kem-BL cells. Two EBV-loss clones of Kem-BL were transfected with pRTS EBER CD2 or with pRTS-CD2 as a control and stable cell lines generated as described for pRTS-CD2 and pRTS EBNA1 CD2. Once again, aliquots of cells were transferred to fresh BL medium or medium containing 50ng/mL or 1000ng/mL dox. After 48 hours, cells were harvested for RNA extraction and EBER1 and EBER2 expression was analysed by QRT-PCR. In parallel, resistance to ionomycin and anti-IgM induced apoptosis was simultaneously measured in matching aliquots of pRTS-CD2 and pRTS EBER CD2 transfected cells using the modified version of the apoptosis assay previously described in section 5.3.2.

Figure 5.20 shows the effect of EBNA1 and EBER expression on resistance of Kem-BL clones to apoptosis inducing agents, ionomycin and anti-IgM. Figure 5.20(A) shows EBER1 and EBER2

expression in EBV-positive Kem-BL clones (kp1 and kp2), EBV-loss Kem-BL clones (kn1 and kn2) and kn1 and kn2 transfected with pRTS-CD2 or pRTS EBER CD2. As expected there was no EBER expression in EBV-loss clones or EBV-loss clones transfected with pRTS-CD2. In Kem-BL clones transfected with pRTS EBER CD2, there was very low but detectable EBER1 and EBER2 expression in both EBV-loss clones in the absence of dox. Addition of 50ng/mL dox caused an increase in EBER1 expression to roughly the level observed in kp1 and kp2 and an increase in EBER2 expression to approximately twice kp1 and kp2 levels. Addition of 1000ng/ml dox increased EBER1 and EBER2 expression a further 2-fold compared to the level observed after treatment with 50ng/ml dox.

Figure 5.20(B) compares the effect on apoptosis of co-expression of EBERs and EBNA1 against EBNA1 expression alone. As previously demonstrated, loss of EBV from Kem-BL cells increased the percentage of cells induced to die by 0.5µg/mL ionomycin from 20% in EBV-positive clones to 50% in EBV-loss clones. This sensitivity of EBV-loss clones to ionomycin was not reversed by the expression of EBNA1 from pRTS-CD2. As observed in clones of Akata-BL, we also found that induced cell death was unaffected by expression of the EBNA1 plus the EBERs. Cell death in pRTS EBER CD2 transfected cells in the absence of dox was very similar to the level observed in EBV-loss clones, despite the low level EBER expression. Induction of EBER expression to the levels observed in EBV-positive Kem-BL clones, kp1 and kp2 with 50ng/mL dox or to approximately twice the level observed in kp1 and kp2 with 1000ng/mL dox was also unable to rescue cells from ionomycin induced cell death.

We found very similar results when we investigated the effect of EBER expression on resistance to anti-IgM induced cell death. Loss of EBV from Kem-BL clones sensitised cells to anti-IgM induced cell death; however expression of EBNA1 alone (from pRTS-CD2) or EBNA1 and EBERs (from pRTS EBER CD2) was unable to reverse this increased apoptosis sensitivity.

Two independent experiments were carried out in both the Akata-BL and Kem-BL background with the same results and we conclude therefore that, contrary to previous reports, EBERs are not responsible for EBV-mediated protection from apoptosis in BL cells. Because of this lack of protection by the EBERs we decided to investigate a cellular gene which was also previously associated with apoptosis in BL cells, the autocrine growth factor, IL-10.

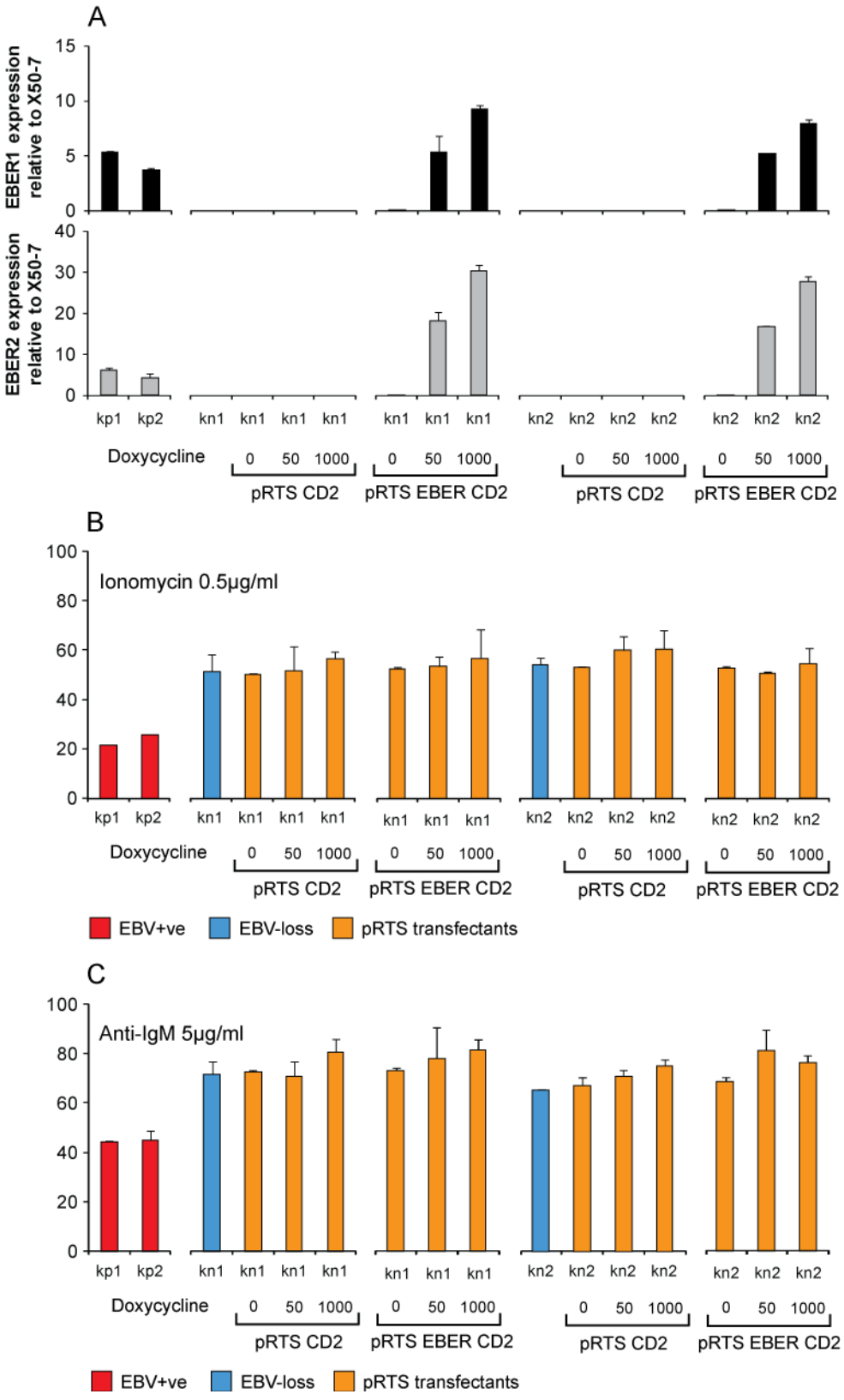


Figure 5.20. Effect of EBNA1 and EBERs on resistance to apoptosis in clones of Kem-BL. (A) Expression of EBER1 and EBER2 in EBV-positive Kem-BL clones, EBV-loss Kem-BL clones and EBV-loss Kem-BL clones transfected with pRTS CD2 and pRTS EBER CD2. EBER expression was determined by QRT-PCR and measured relative to the X50-7 LCL. (B and C) Effect of EBNA1 and EBERs on resistance of clones of Kem-BL to cell death induced by 0.5µg/ml ionomycin or 5µg/ml Anti-IgM. Cell death in cells induced with dox was calculated as the percentage of GFP-positive cells induced to die. For those cells not induced with dox total cell death was recorded. Error bars represent standard deviation in cell death across 3 parallel wells from the same experiment.

5.4 The role of IL-10 in the apoptosis resistant phenotype of BL cells

5.4.1 IL-10 expression in EBV-positive and EBV-loss BL clones

IL-10 is an autocrine growth factor, which is secreted by activated lymphocytes. It acts as a paracrine signalling agent for surrounding B cells, where it enhances cell survival, proliferation, and antibody production and modulates expression of Th1 cytokines, MHC class II and the NF-kappa B and JAK-STAT signalling pathways. In BL cells IL-10 has been reported as a mechanism of EBV-mediated apoptosis resistance and has been associated with EBER expression (Kitagawa et al., 2000; Samanta et al., 2008); thus, in the absence of an apoptotic effect from EBNA1 or EBERs, we decided to investigate IL-10 expression in the panel of EBV-positive and EBV-loss clones.

To determine IL-10 expression we used QRT-PCR to analyse IL-10 and IL-10 receptor (IL-10R) expression in paired EBV-positive and EBV-loss clones of Akata-BL, Awia-BL, Mutu-BL and Eli-BL. RNA was extracted from BL cell lines and reverse transcribed to cDNA using random primers. IL-10 and IL-10R expression was then measured relative to a 2089 virus transformed LCL using commercial Taqman expression assays and the results are shown in Figure 5.21.

In general the expression of IL-10 in all the BL clones was low compared to the expression in the 2089 LCL control cell line. Expression in clones of Akata-BL, Awia-BL and Eli-BL was less than 20% of the level observed in 2089 LCL and there was little difference between EBV-positive and EBV-loss clones. The one exception where we did find increased IL-10 expression in EBV-positive clones relative to EBV-loss clones was in Mutu-BL. IL-10 in EBV-positive Mutu-BL clones was found to be between 2 and 8 times the level observed in 2089 LCL, whereas there was little or no IL-10 expression in EBV-loss Mutu-BL clones. Although it is possible that these differences in IL-10 expression could contribute to the altered apoptosis resistance between EBV-positive and EBV-loss Mutu-BL clones, it is unlikely that it is mediated by EBV as restoration of Latency I viral gene expression in reinfected Mutu-BL clones, r1 and r2 did not increase IL-10 above the low level seen in EBV-loss clones. This is despite the fact that reinfected Mutu-BL clones, r1 and r2 demonstrated the same resistance to ionomycin induced apoptosis as EBV-positive Mutu-BL clones.

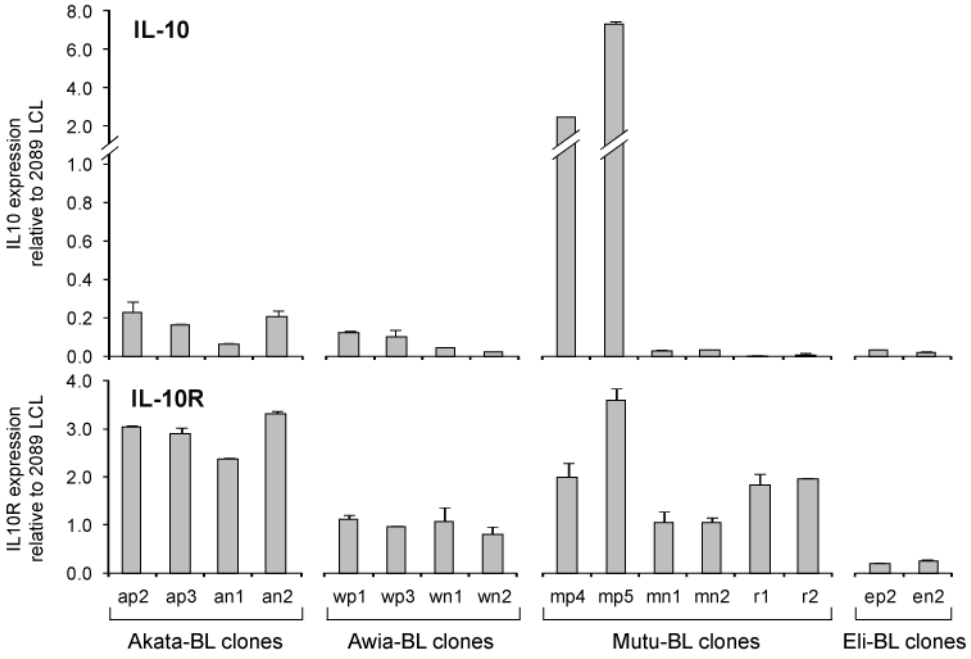


Figure 5.21. IL-10 and IL-10 receptor (IL-10R) expression in EBV-positive and EBV-loss clones of Akata-BL, Awia-BL, Mutu-BL and Eli-BL. Expression was determined using specific QRT-PCR assays and was measured relative to a 2089 virus transformed LCL (assigned a value of 1).

When we examined expression of IL-10R in BL cells we found detectable expression in all BL clones, ranging from 20% of the level to 3 times the level observed in 2089 LCL. Just as for IL-10, we found no difference in IL-10R expression between EBV-positive and EBV-loss clones except for a small difference in clones of Mutu-BL. Loss of EBV in Mutu-BL appeared to cause approximately a 2-fold reduction in IL-10R expression and this appeared to be partially restored by reinfection by EBV in clones r1 and r2, but the significance of this result is not currently understood. Overall, though, it appears that EBV does not generally directly regulate IL-10 or IL-10R expression in BL cells. This contradicts the work done in the Akata-BL where IL-10 was suggested as a major contributor to EBV-mediated apoptosis resistance (Kitagawa et al., 2000; Samanta et al., 2008).

5.4.2 Effect of EBERs on IL-10 expression

To thoroughly investigate any possible link between EBERs and IL-10 expression we next determined IL-10 expression in EBV-loss clones of Akata-BL transfected with pRTS EBER CD2. As reference, Figure 5.22(A) shows EBER expression in EBV-positive clones, EBV-loss clones and EBV-loss clones transfected with pRTS-CD2 or pRTS EBER CD2. As previously shown in Figure 5.19, transfection of Akata-BL EBV-loss clones with pRTS EBER CD2 causes low level EBER1 and EBER2 expression. Activation of pRTS EBER CD2 with 50ng/ml dox induced EBER1 expression similar to the level observed in EBV-positive Akata-BL clones and higher EBER2 expression, while addition of 1000ng/mL dox induced levels of both EBER1 and EBER2 to higher than is observed in EBV-positive Akata-BL clones.

Figure 5.22(B) shows the IL-10 expression in the same transfected Akata-BL clones. There is little or no difference in IL-10 expression between EBV-positive and EBV-loss Akata-BL clones and expression of EBNA1 via transfection of pRTS-CD2 has no effect on the levels of IL-10. Interestingly transfection of pRTS EBER CD2 also has no effect on IL-10 expression even after dox induction of high levels of EBERs. This lack of EBER-mediated IL-10 induction may help to explain why EBERs seem unable to rescue these Akata-BL cells from drug induced cell death.

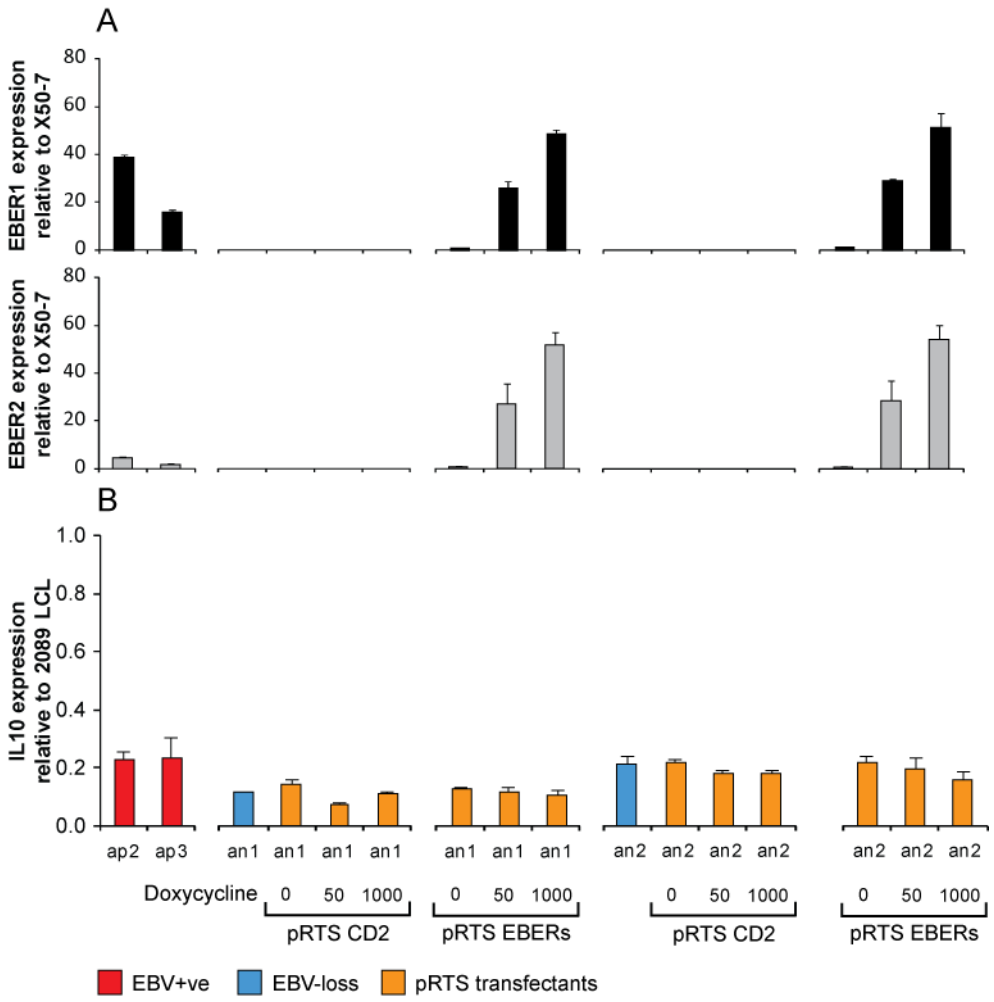


Figure 5.22. Effect of EBNA1 and EBERs on cellular IL-10 expression in clones of Akata-BL. (A) Expression of EBER1 and EBER2 in EBV-positive, EBV-loss and EBV-loss clones of Akata-BL transfected with pRTS vectors. (B) Cellular IL-10 expression as determined by QRT-PCR measured relative to a 2089 virus transformed LCL (assigned a value of 1).

5.5 Viral IL-10 expression in EBV-positive and EBV-loss BL clones

During late lytic cycle, EBV expresses a homologue of IL-10 (vIL-10) to increase cell viability. vIL-10 displays extensive homology with the cellular human IL-10 gene (hIL-10) (Moore et al., 1990). As shown by the alignment in Figure 5.23(A), there is a significant degree of overlap between the hIL-10 mRNA sequence and the vIL-10 sequences of Ag87 and B95-8, especially beyond the 5' end of gene. The similarity between the aa sequence of hIL-10 and vIL-10 is even more striking especially past the divergent N-terminal region. The overall colinear aa sequence identity to hIL-10 is nearly 90%. Therefore, as a control in the investigation of IL-10 in EBV-positive and EBV-loss BL clones, Dr Andrew Bell developed a QRT-PCR specific for vIL-10. Specificity of detection was ensured by placing the primers and probe within the unique 3' end shown in Figure 5.23(A). RNA samples from EBV-positive and EBV-loss clones were DNase I treated as the vIL-10 transcript is unspliced and it is possible that contaminating EBV genomes in the RNA samples could be detected by vIL-10 primers. Samples were then reverse transcribed into cDNA using random primers and analysed by QRT-PCR.

The expression of IL-10 in EBV-positive and EBV-loss clones of Akata-BL, Awia-BL, Mutu-BL and Eli-BL (which was shown previously in Figure 5.21) is repeated in Figure 5.24(A) as reference for the expression of vIL-10, which is shown in Figure 5.24(B). Interestingly, there were detectable levels of viral IL-10 in all EBV-positive BL clones, although the expression pattern is different to the pattern observed in IL-10. Therefore it appears unlikely that there is crossover between the two assays. Compared to the reference cell line IMI05, which has around 2% of cells in lytic cycle, vIL-10 was around 60% in EBV-positive Akata-BL clones, 40% in EBV-positive Awia-BL clones, 20% in EBV-positive Mutu-BL clones and was also detectable at low levels in reinfected clones of Mutu-BL and the EBV-positive clone of Eli-BL. Interestingly, vIL-10 expression in these clones is slightly higher than would be expected from the low level F-U and BRLF1 expression discovered during the initial investigation of EBV viral latency, which was typically around 20% of the level observed in a lytic LCL. Expression of most lytic cycle genes would be highly unlikely to affect the overall survival of cell cultures because their expression is limited to a small number of cells. However vIL-10 is secreted from cells into the surrounding medium; thus it is feasible that vIL-10 produced by a small number of lytically active cells may be able to promote apoptosis protection in the remaining culture.

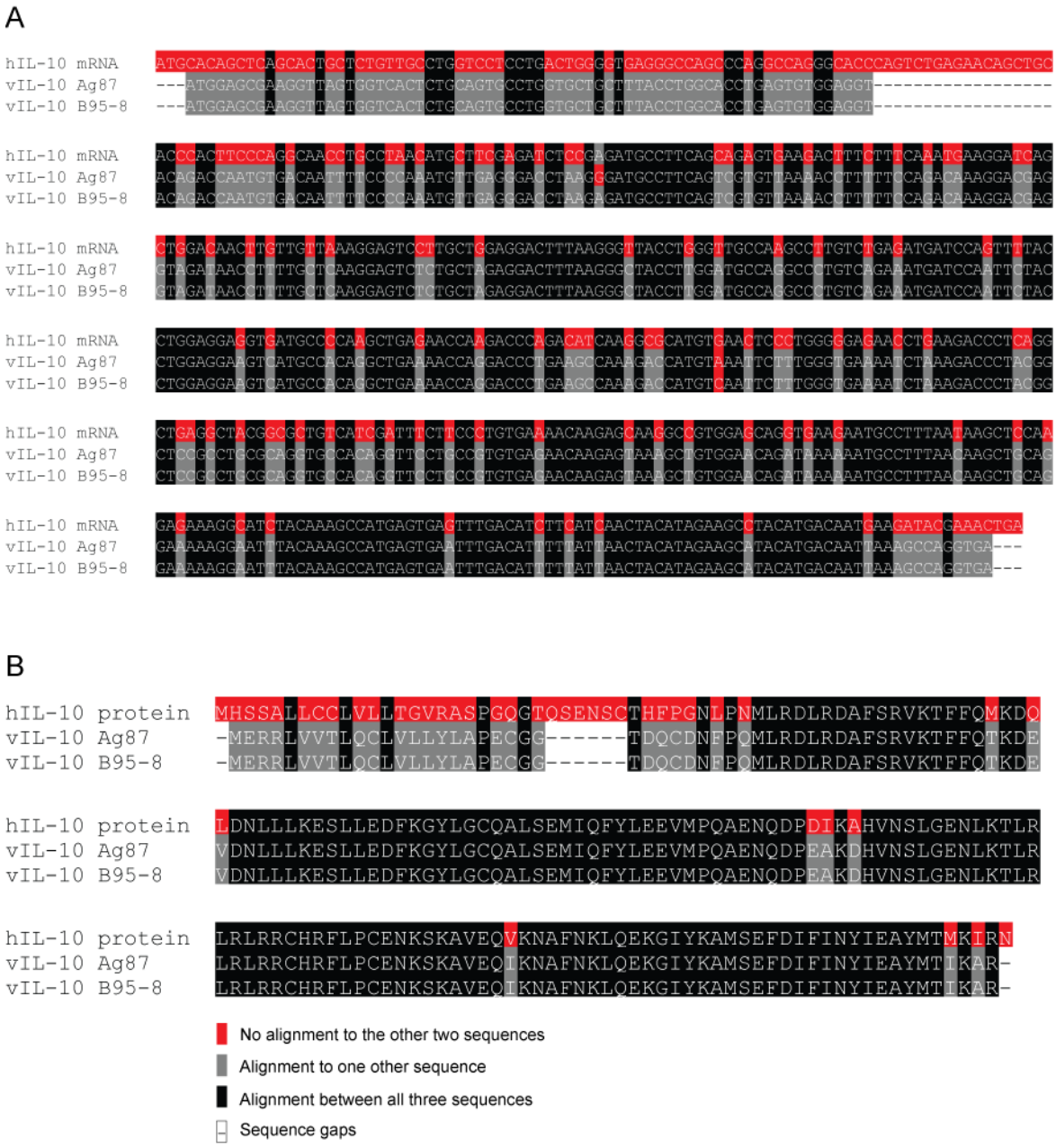


Figure 5.23. Alignment of cellular human IL-10 (hIL-10) to vIL-10 from Ag87 and B95-8 EBV strains. (A) Alignment of hIL-10 mRNA to vIL-10 gene sequences. (B) Alignment of hIL-10 protein to vIL-10 protein sequences.

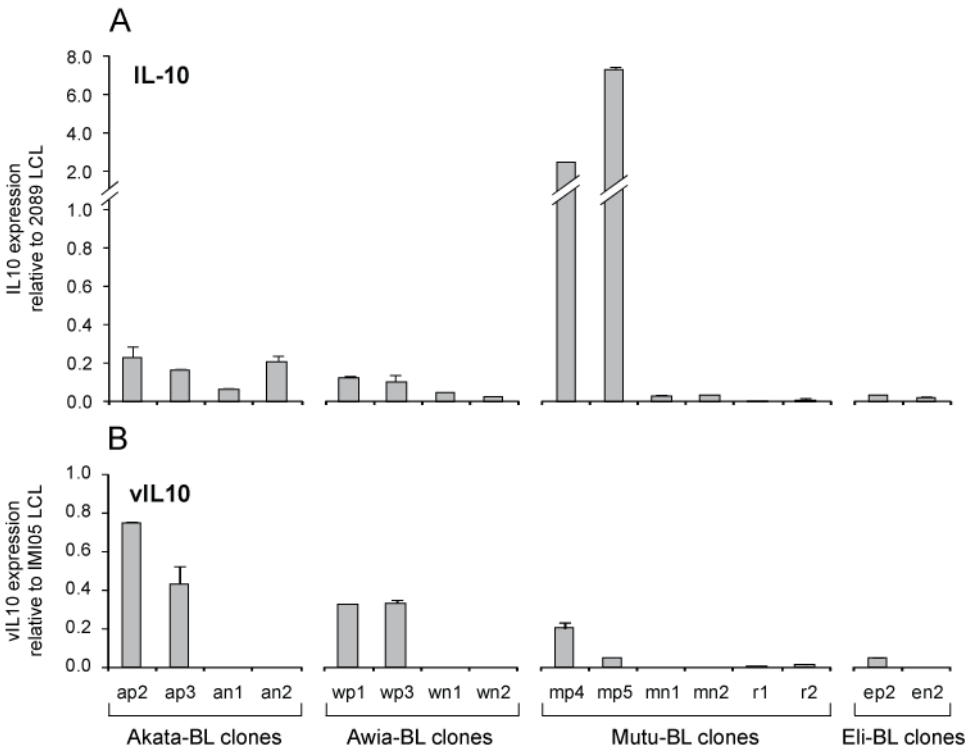


Figure 5.24. Expression of cellular IL-10 and the EBV viral IL-10 homologue (vIL-10), in EBV-positive and EBV-loss clones of Akata-BL, Awia-BL, Mutu-BL and Eli-BL, as determined by quantitative RT-PCR. (A) Expression of cellular IL-10. Data has already been shown in Figure 5.21, but is repeated as reference for (B) the expression of vIL-10. Values were measured relative to an EBV transformed LCL with 2% of cells in lytic cycle (assigned a value of 1).

5.5.1 EBV lytic cycle expression in EBV-positive and EBV-loss BL clones

Since we found evidence of vIL-10 expression in EBV-positive BL clones during screening of clones for IL-10 and IL-10R, we decided to further characterise EBV lytic cycle activity in more detail by examining a range of lytic cycle genes. We aimed to discover whether vIL-10 expression in BL cells was part of a broader activation of the EBV lytic cycle or if vIL-10 could be expressed in latently infected cells by a novel mechanism.

Many of the lytic cycle QRT-PCR assays measure the expression of unspliced viral transcripts, so RNA samples from BL cell lines were DNase I treated to removed contaminating EBV genomes, then reverse transcribed to cDNA using random primers. Along with the QRT-PCR assays for vIL-10 and the immediate early lytic cycle genes, BRLF1 and BZLF1 which have previously been described, we also investigated expression of the EBV early lytic cycle genes, BMLF1, BHRF1, BNLF2a, BALF1 and BALF4 and the late lytic cycle genes BVRF2 and gp350. Expression of these lytic cycle genes was measured relative to the spontaneously permissive LCL control cell line IMI05, which has around 2% of cells in active lytic cycle.

The results of these QRT-PCR assays are shown in Figure 5.25. As expected we found no evidence of EBV lytic cycle transcripts in any EBV-loss clones. We found little or no expression of BZLF1 in EBV-positive BL clones; however in clones of Akata-BL we believe that this is due in part to a polymorphism, which reduces the efficiency of the QPCR reaction. Interestingly, there was expression of the other immediate early lytic cycle gene, BRLF1 in all EBV-positive clones. BRLF1 expression varied between 10-40% of the level seen in IMI05 in EBV-positive clones of Akata-BL, Awia-BL and Mutu-BL, although expression in reinfected clones of Mutu-BL and the EBV-positive clone of Eli-BL was very low.

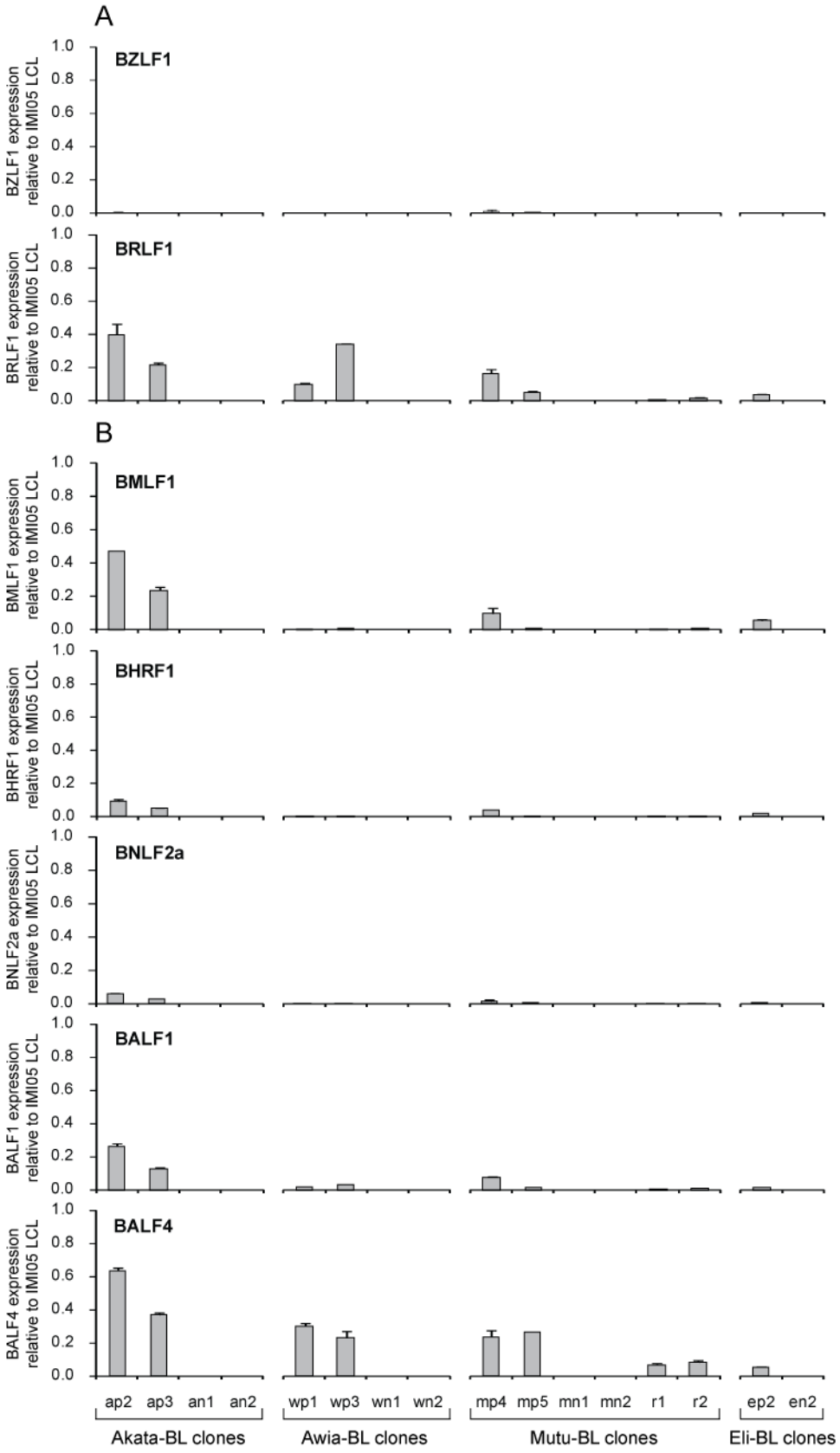
Expression of EBV early lytic cycle genes was low in most BL clones. BMLF1 could be detected in clones of Akata-BL at around 30% of the level observed in IMI05; however expression could not be detected in Awia-BL clones and there was only low level expression in EBV-positive clones of Mutu-BL and Eli-BL. Expression of BNLF2a and the viral Bcl-2 homologue, BHRF1 was very low in all BL lines. The highest BNLF2a and BHRF1 expression was found in clones of Akata-BL, but this was still less than 10% of the level observed in IMI05. BALF1 expression was observed at around 20% of the level of IMI05 in EBV-positive clones of Akata-BL, but was very low in all other BL clones. The most

abundantly expressed early lytic cycle gene was BALF4. We found BALF4 expression at around 40% of IMI05 levels in EBV-positive clones of Akata-BL, Awia-BL and Mutu-BL and at around 10% of IMI05 levels in reinfected Mutu-BL clones and EBV-positive Eli-BL.

Interestingly, there was higher expression of the EBV late lytic cycle genes than the immediate early or early lytic cycle transcripts. As previously described, expression of vIL-10 was observed in all EBV-positive clones. Compared to IMI05, BVRF2 expression was around 60% in EBV-positive Akata-BL clones, 10% in EBV-positive Awia-BL clones, 20% in EBV-positive Mutu-BL clones, 60% in reinfected Mutu-BL clones and 10% in EBV-positive Eli-BL. Finally gp350 expression varied between 10-50% of IMI05 levels in EBV-positive BL clones, but expression was almost absent in reinfected Mutu-BL clones.

Overall the results of QRT-PCR analysis of lytic cycle activity in EBV-positive BL clones indicates that although there is little expression of the major lytic cycle activator, BZLF1, low level activity of the EBV lytic cycle may be being driven by the other immediate early lytic cycle gene, BRLF1. Expression of most of the early lytic cycle genes is low, but interestingly there is significant expression of the glycoprotein gp110 gene (BALF4) and all of the late lytic cycle genes. This indicates that, although vIL-10 expression is higher than would be expected from expression of the immediate early lytic cycle genes, it is likely to be expressed along with other EBV late lytic cycle genes as part of the normal EBV lytic cycle.

Initially it would appear unlikely that EBV lytic cycle genes could contribute to resistance to apoptosis as there is low expression of the Bcl-2 homologues, BHRF1 and BALF1. However it is still possible that the observed levels of vIL-10 could contribute to apoptosis resistance because, like cellular IL-10, vIL-10 is released from cells into the culture medium where it can stimulate the IL-10 receptors of neighbouring BL cells.


A
BZLF1
BZLF1 expression relative to IMI05 LCL
BRLF1
BRLF1 expression relative to IMI05 LCL
B
BMLF1
BMLF1 expression relative to IMI05 LCL
BHRF1
BHRF1 expression relative to IMI05 LCL
BNLF2a
BNLF2a expression relative to IMI05 LCL
BALF1
BALF1 expression relative to IMI05 LCL
BALF4
BALF4 expression relative to IMI05 LCL
0.0
0.2
0.4
0.6
0.8
1.0
ap2 ap3 an1 an2
wp1 wp3 wn1 wn2
mp4 mp5 mn1 mn2 r1 r2
ep2 en2
Akata-BL clones
Awia-BL clones
Mutu-BL clones
Eli-BL clones

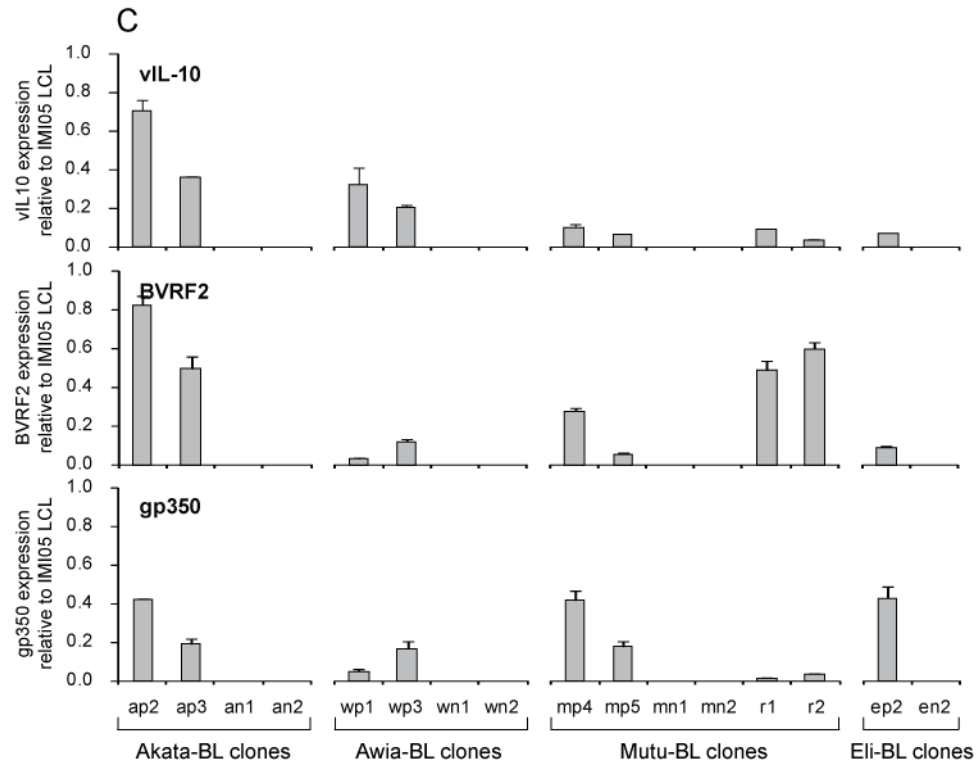


Figure 5.25. Expression of EBV lytic cycle genes in BL clones. QRT-PCR analysis of (A) immediate early lytic cycle genes, (B) early lytic cycle genes and (C) late lytic cycle genes in EBV-positive and EBV-loss clones of Akata-BL, Awia-BL, Mutu-BL and Eli-BL. Transcription levels are measured relative to an EBV transformed LCL with 2% of cells in lytic cycle (assigned a value of 1).

5.6 Effect of inhibition of EBV lytic cycle on resistance to apoptosis

In order to test if expression of vIL-10 could be responsible for the apoptosis resistance seen in EBV-positive clones, cells were treated with ACV to block lytic cycle activity before carrying out an apoptosis assay.

Aliquots of 2 EBV-positive and 2 EBV-loss clones of Akata-BL, Awia-BL and Mutu-BL were grown in normal BL medium or BL medium supplemented with 200μM ACV for 2 weeks to inhibit viral replication. Cells were then harvested for analysis of EBV lytic cycle activity by QRT-PCR. A parallel aliquot of each clone was also induced into cell death using 0.5μg/mL ionomycin and the percentage of cells induced into apoptosis and necrosis was determined by Syto 16 and propidium iodide staining.

Figure 5.26(A) shows the expression of the EBV late lytic cycle antigens, vIL-10 and gp350 in BL clones after 2 weeks culture in normal BL medium or medium supplemented with 200μM ACV. Treatment with ACV caused a reduction in expression of vIL-10 and gp350 in EBV-positive BL clones of Akata-BL, Awia-BL and Mutu-BL to around 15% of the level observed in normal BL medium.

Figure 5.26(B) shows resistance to ionomycin induced cell death in EBV-positive and EBV-loss Akata-BL, Awia-BL and Mutu-BL clones grown in normal media or ACV-supplemented medium. Growth in ACV appeared to have no effect on resistance to apoptosis in EBV-positive or EBV-loss clones of Akata-BL, Awia-BL or Mutu-BL; thus a reduction in vIL-10 expression does not sensitise EBV-positive cells to ionomycin induced cell death. However as treatment with ACV does not totally abolish vIL-10 activity, low level expression of vIL-10 cannot be ruled out as a possible source of protection from ionomycin induced cell death.

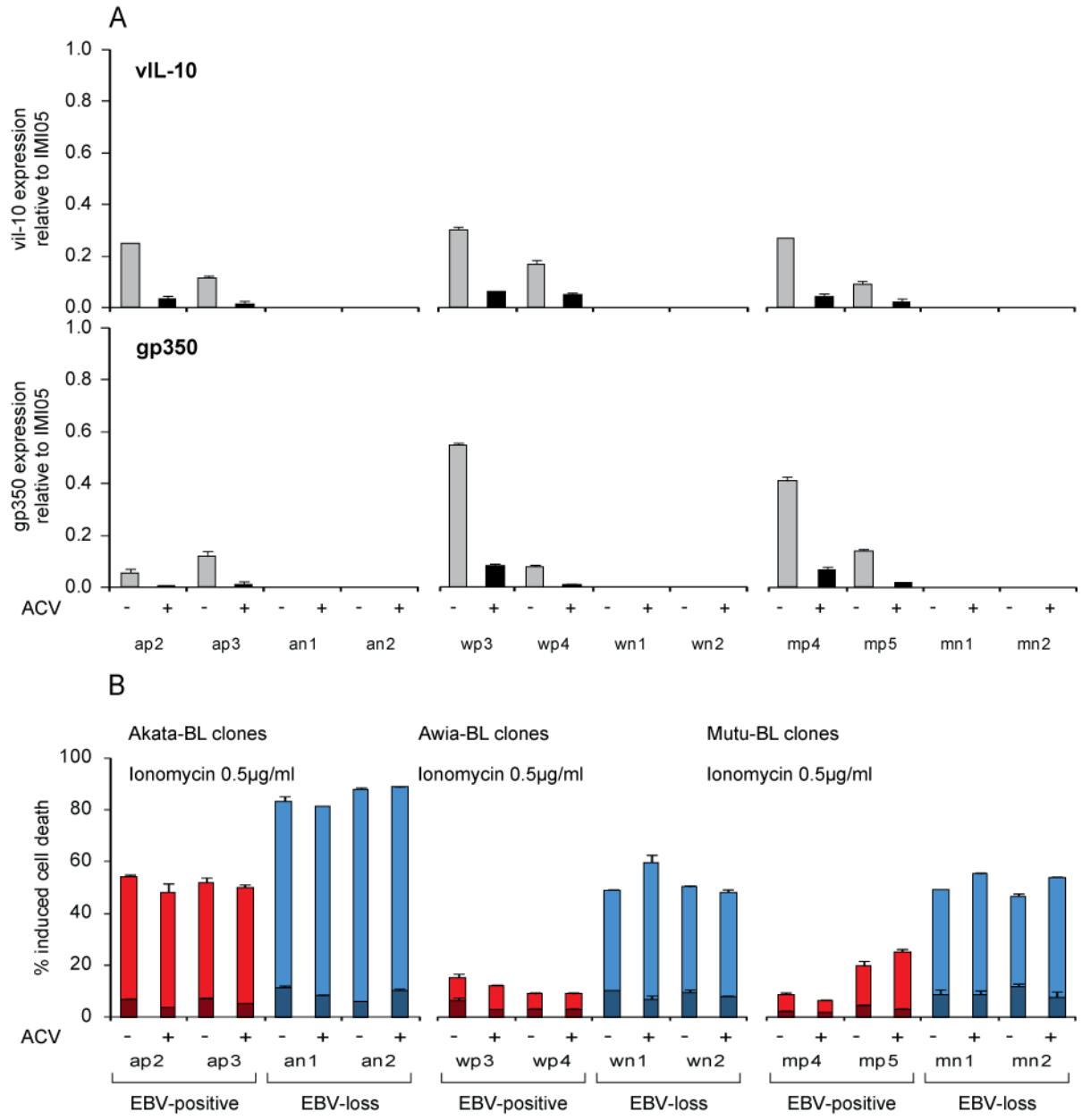


Figure 5.26. The effect of inhibition of the EBV lytic cycle with Acyclovir (ACV) on resistance to apoptosis in clones of Akata-BL, Awia-BL and Mutu-BL. (A) Expression of EBV late lytic cycle genes, vIL-10 and gp350 in EBV-positive and EBV-loss clones grown in BL medium or BL medium+200mM ACV as determined by QRT-PCR. Transcription levels were measured relative to an EBV transformed LCL with 2% of cells in lytic cycle (assigned a value of 1). (B) Apoptosis resistance in EBV-positive and EBV-loss clones of Akata-BL, Awia-BL and Mutu-BL maintained in BL medium or BL medium+200mM ACV. Cell death was determined by flow cytometry after staining with Syto 16 and propidium iodide. The lighter coloured section of each bar represents induced apoptosis and the darker section is induced necrosis. Error bars represent standard deviation in cell death across 3 parallel wells from the same experiment.

5.7 Conditioning EBV-loss clones with media from EBV-positive clones

In a parallel experiment we conditioned EBV-loss clones with the medium in which EBV-positive clones had been growing. If apoptosis resistance was due to paracrine vIL-10 signalling from a small number of lytically replicating cells, then growing EBV-loss clones in medium from EBV-positive clones should increase resistance to apoptosis.

To test this supposition we used a modified version of the standard apoptosis assay. The supernatant was isolated from 2 EBV-positive and 2 EBV-loss clones of Akata-BL by 2x centrifugation at 1,400 RPM for 4 minutes. Supernatant from EBV-positive and EBV-loss clones was then mixed 1:1 with normal BL medium to make 'Akata EBV-positive' conditioned medium and 'Akata EBV-loss' conditioned medium. $1.5x10^4$ cells from 2 EBV-positive and 2 EBV-loss Akata-BL clones were then transferred to multiple wells of 96 well plates in 200µL of 'Akata EBV-positive' conditioned medium or 'Akata EBV-loss' conditioned medium and incubated for 24 hours. 100µL of medium was then removed and replaced with 100µL of 2x apoptosis inducing agent (ionomycin or roscovitine), in the appropriate conditioned medium. Cell death was induced for 48 hours and apoptosis and necrosis determined by Syto 16 and propidium iodide staining. The process was repeated in EBV-positive and EBV-loss clones of Awia-BL and Mutu-BL. Note that the Awia-BL clones were incubated with supernatant from Awia-BL clones and Mutu-BL clones were incubated with supernatant from Mutu-BL clones and that cell death was determined using ionomycin and anti-IgM cross linking.

As shown in Figure 5.27(A), the percentage of ionomycin induced cell death in each clone was not affected by whether it had been conditioned in medium from EBV-positive or EBV-loss clones. The exact percentage of induced cell death in this experiment was slightly different to other apoptosis assays using the same BL clones, but this is likely to be due to the slightly different method used to induce apoptosis.

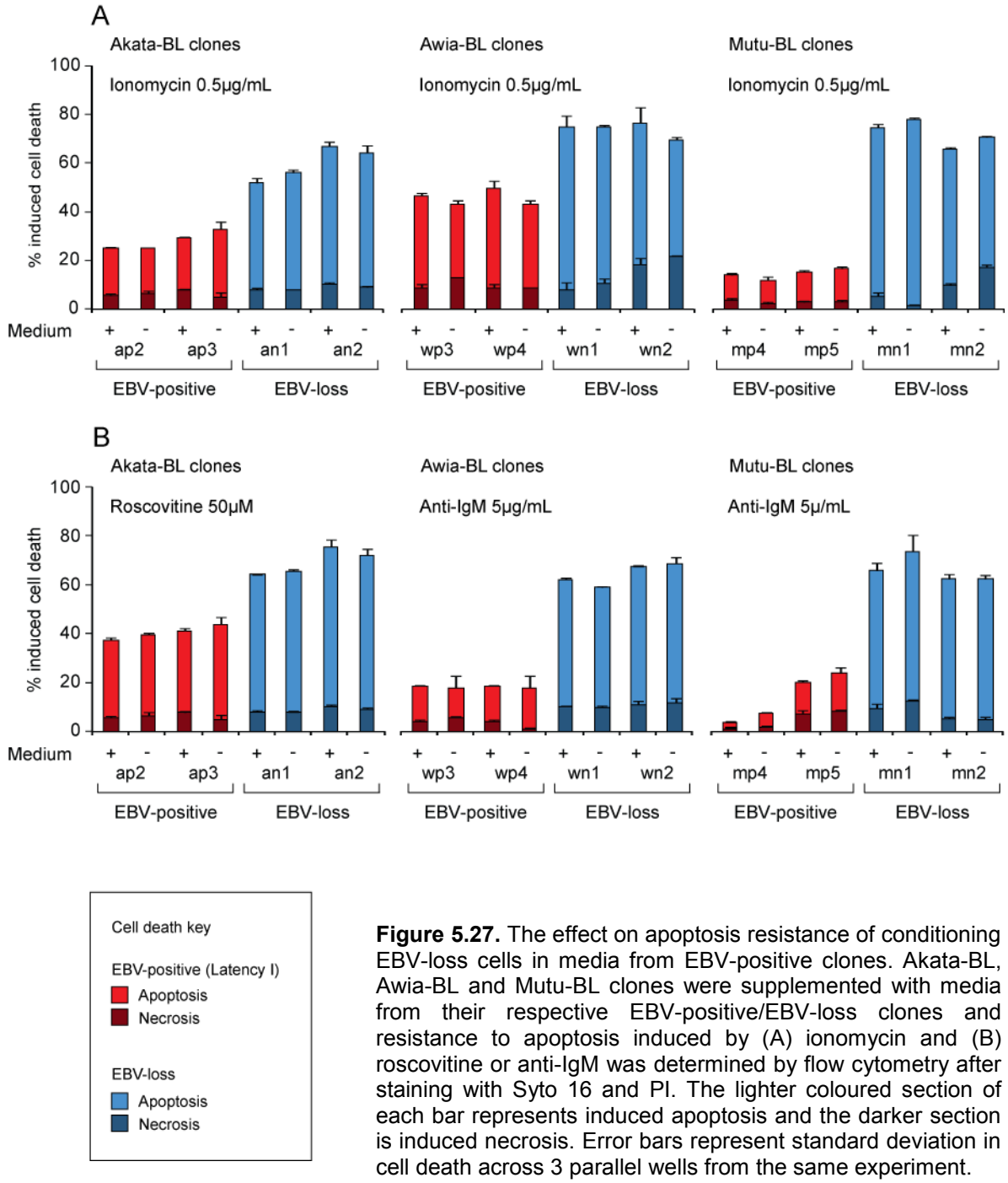


Figure 5.27. The effect on apoptosis resistance of conditioning EBV-loss cells in media from EBV-positive clones. Akata-BL, Awia-BL and Mutu-BL clones were supplemented with media from their respective EBV-positive/EBV-loss clones and resistance to apoptosis induced by (A) ionomycin and (B) roscovitine or anti-IgM was determined by flow cytometry after staining with Syto 16 and PI. The lighter coloured section of each bar represents induced apoptosis and the darker section is induced necrosis. Error bars represent standard deviation in cell death across 3 parallel wells from the same experiment.

5.8 Effect of vIL-10 on apoptosis in EBV-loss clones of Akata-BL

To supplement the above investigations into the effect of vIL-10 on apoptosis resistance in BL cells, the vIL-10 gene was cloned into the pRTS CD2 backbone in the same manner as the EBERs (Figure 2.6). This final section of work was carried out in our lab during the writing of this thesis predominantly by Wendy Thomas. In a preliminary set of experiments, pRTS CD2 and pRTS vIL-10 CD2 were transfected into the Akata-BL EBV-loss clone, an1. Transfected cells were induced with 50ng/ml dox for 48 hours and both vector positive and vector negative cells were isolated using FACS in the same manner as has previously been used for Kem-BL clones after transfection with pRTS CD2 vectors (section 2.17.1).

Next vIL-10 expression upon the addition of dox was examined in each sorted population using QRT-PCR. As shown in Figure 5.28(A), the GFP-positive pRTS vIL-10 CD2 transfected cells expressed vIL-10 at several hundred times the level observed in Akata-BL. When we investigated the effect of vIL-10 expression on apoptosis (Figure 5.28(B)), we found that, in accordance with the work on vIL-10 described above, vIL-10 expression was unable to increase the apoptosis resistance of EBV-loss clones. It would be informative to examine the effect of vIL-10 expression in additional EBV-loss clones from both Akata-BL and Kem-BL; however it appears from these preliminary results that vIL-10 is not the agent responsible for apoptosis resistance in EBV-positive BL cells.

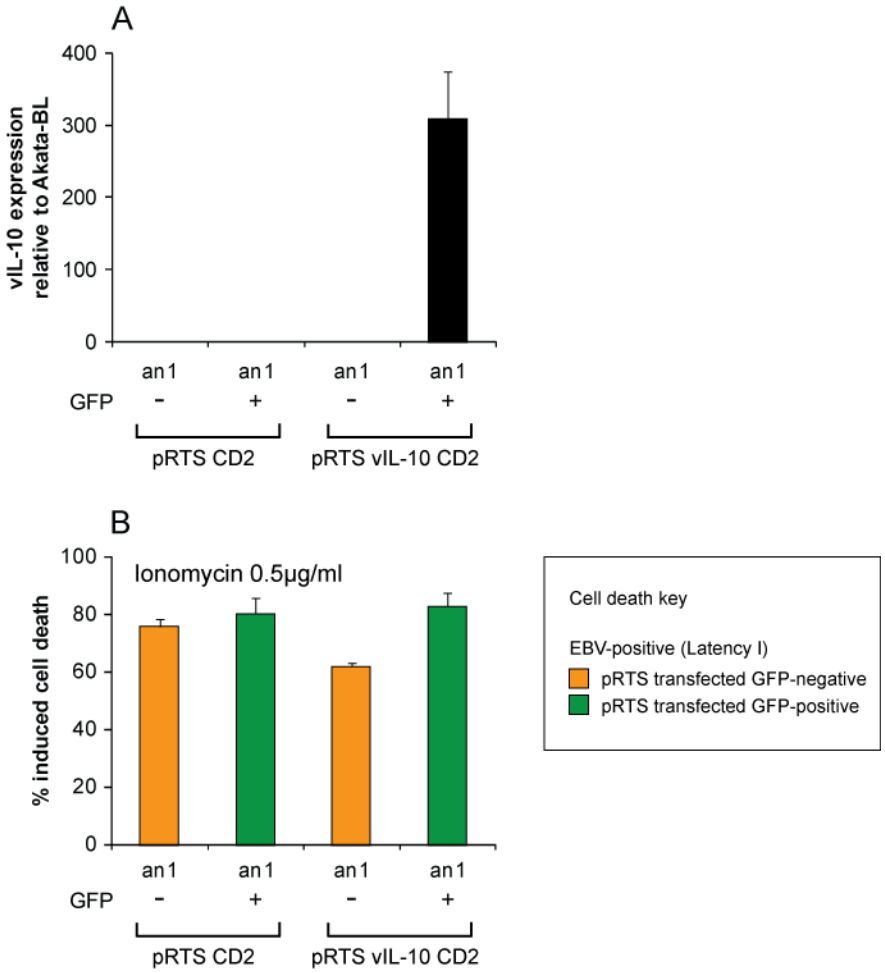


Figure 5.28. Effect on apoptosis resistance of EBNA1 and vIL-10 expression in an EBV-loss clone of Akata-BL. (A) vIL-10 expression in pRTS CD2 and pRTS vIL-10 CD2 transfected an1 cells as determined by QRT-PCR and measured relative to Akata-BL. (B) Effect of EBNA1 and vIL-10 expression on resistance to apoptosis induced by 0.5µg/ml ionomycin. Cell death in the GFP-positive population was calculated as the percentage of GFP-positive cells induced to die. In GFP-negative populations total cell death was recorded. Error bars represent standard deviation in cell death across 3 parallel wells from the same experiment.

Discussion III

(a) Use of recombinant viruses to restore apoptosis resistance

Initially we aimed to formally demonstrate that EBV was the agent responsible for apoptosis resistance in EBV-positive clones by reinfecting EBV-loss clones with a recombinant EBV. However we encountered three major obstacles to this investigation. Firstly we found that only clones of Mutu-BL, Awia-BL and Eli-BL were infectable with recombinant EBV. We were unable to infect EBV-loss clones of Akata-BL, which may be due to the low levels of the EBV receptor (CD21) on the surface of Akata-BL cells (Evans et al., 1995). In the published reports describing reinfection of Akata-BL, EBV-loss clones were either infected with EBV, cloned at ~10,000 cells/well and large numbers of clones drug selected to isolate rare infected cells (Komano et al., 1998) or the virus was delivered through repeated rounds of infection (Ruf et al., 1999). Use of these methods implies that infection of Akata-BL cells is a very rare event. It is possible that we could improve the efficiency of Akata-BL infection if cells were cultivated with virus producing cell lines such as 293 cells carrying the B95-8 BAC or if cells were transfected with a CD21 expression vector; however we did not pursue this avenue of investigation and relied instead on reinfecting the remaining EBV-loss clones.

Secondly we found that without drug selection, most cells reinfected with recombinant EBV were unable to maintain a stable EBV infection. This characteristic could stem from the manner in which EBV-loss clones were formed and implies that EBV-loss cells are in some way predispositioned to lose EBV. As discussed in Chapter 1, this could be due to genetic changes which reduce the stability of the EBV infection or result in some incompatibility with viral gene expression. Such changes would also prevent the reintroduction of a stable EBV infection within these cell lines.

The third barrier to this investigation was that, in the vast majority of reinfected clones, the incoming recombinant EBV established a Latency III pattern of viral gene expression. We observed this phenomenon both when reinfected cells were single cell cloned on to fibroblast feeder layers and when infected cultures were drug selected to prevent spontaneous EBV-loss. This finding is in agreement with infection studies carried out in EBV-negative BL cell lines including BL28, Ramos and DG75 where Latency III infection was always observed (Trivedi et al., 2001). In addition superinfection

of EBV-positive Akata-BL cells leads to Latency III gene expression from the incoming virus, while the resident virus remains in latency I (Evans et al., 1995). Our experience however contradicts previous reports, where infection of EBV-loss clones of Akata-BL and Mutu-BL with recombinant EBV restored Latency I infection (Kitagawa et al., 2000; Komano et al., 1998; Ruf et al., 1999). Latency I reinfections in these studies were achieved using either the cloning method (Komano et al., 1998) or the repeated infection method (Ruf et al., 1999) described above and these alternative approaches to infection could be the reason for the different pattern of gene expression.

The predominance of the Latency III infection in our study is perhaps unsurprising however, given the normal EBV biology of B cell infection. Infection of resting B cells *in vitro* always results in Latency III gene expression (Alfieri et al., 1991). Latency I infection is believed to represent a strategy to escape immune surveillance and enter the memory B cell compartment; however *in vitro* no such drive to down-regulate Latency III gene expression exists. Indeed, the Cp and Wp promoters of EBV genomes in cells with restricted Latency I and Latency II infection have been shown to be heavily methylated (Schaefer et al., 1997), while these promoters are unmethylated in freshly infected B lymphocytes (Hutchings et al., 2006).

Despite these difficulties, after a significant number of reinfection experiments we found one drug selected reinfection of EBV-loss Mutu-BL which appeared to express both Latency I and Latency III transcripts, indicating that it contained a mixture of cells in both forms of viral latency. When this mixed Mutu-BL population was single cell cloned by limiting dilution, we were able to generate a panel of Latency I reinfected clones. Importantly we found that reinfection had restored resistance to apoptosis in all the reinfected clones which strongly indicates that the difference in apoptosis resistance between EBV-positive and EBV-loss clones is caused by EBV. To demonstrate this beyond reasonable doubt would require more reinfected clones not just on Mutu-BL, but also on other BL backgrounds. A further limitation of the experiment is that the drug selection procedure may have contributed to the increased resistance to apoptosis.

The rarity with which Latency I infections can be re-established *in vitro* is currently unexplained. However it may reflect the dominance of latency III infection, once established *in vitro*, not just over Latency I but also over the c-myc-driven growth programme with which Latency III appears to be incompatible (Polack et al., 1996; Schuhmacher et al., 1999; Pajic et al., 2001). It is possible that

Latency I reinfections could be more consistently produced with a virus that was defective in both Cp and Wp initiated transcription; this possibility will be investigated in our laboratory once the relevant mutant EBV construct has been generated. In the meantime, we pursued the final set of experiments on the assumption (based on results in this Chapter and in the first Chapter of results) that Latency I infection was indeed directly responsible for the differences observed between EBV-positive and EBV-loss BL cells in apoptosis assays.

(b)The role of individual viral gene products in apoptosis resistance

In the next part of this investigation we set out to determine whether any of the individual viral gene products associated with Latency I was responsible for the apoptosis resistance phenotype. To this end we used the episomally maintained EBNA1/ori-P pRTS vector as a doxycycline-regulated expression system. Genes of interest were cloned into the pRTS backbone and their expression controlled by titrating doxycycline (dox) into the cell medium. In this system transfected cells are enriched using MACS or FACS, thereby negating the need for drug selection. Furthermore, because the cultures this produced contain both vector-positive and vector-negative cells, all assays on test cultures have their own internal controls.

(i) EBNA1

We first analysed the effect of EBNA1 expression, as EBNA1 is the only viral protein expressed in Latency I and there is a wealth of evidence incriminating EBNA1 as a possible mediator of cellular change. Thus EBNA1 expression appears to promote B cell lymphoma in transgenic mice (Wilson and Levine, 1992; Wilson et al., 1996), though this observation remains controversial (Kang et al., 2008). In EBV-positive BL cell lines including Mutu-BL, the inhibition of EBNA1 expression using a dominant negative EBNA1 or anti-EBNA1 siRNA appeared to retard cell growth; however this effect appeared to be due to the loss of EBV genomes from infected cells (Nasimuzzaman et al., 2005; Yin and Flemington, 2006). In one study however, inhibition of EBNA1 was found to inhibit the survival of BL cells in the absence of genome loss (Kennedy et al., 2003), while expression of EBNA1 in WT p53 epithelial cell lines blocked apoptosis induced through transfection of a p53 expression vector. In osteosarcoma cells EBNA1 expression also provided protection against apoptosis induced via UV

irradiation, which may result from the binding of EBNA1 to the p53 stabilising protein USP7 (Saridakis et al., 2005). Interestingly however, an anti-apoptotic role for EBNA1 has not been shown in B lymphocyte-derived cell lines.

By using various dox concentrations we expressed EBNA1 in EBV-loss clones on two BL backgrounds, both at levels comparable to EBV-positive clones and at significantly higher levels (Figure 5.11 and Figure 5.12). When we evaluated the effect on apoptosis resistance (Figure 5.14 and Figure 5.15), we found that EBNA1 expression (even at levels several times higher than in an EBV-positive clone) was unable to restore apoptosis resistance to EBV-loss clones. Although contrary to the reports noted above, this result is in agreement with several independent studies, which found that EBNA1 was unable to restore resistance to apoptosis to EBV-loss BL clones (Komano et al., 1998; Ruf et al., 1999). The discrepancies between these studies and the work described in the previous paragraph may reflect the different cell background in these experiments. Thus EBNA1-mediated apoptosis protection in non-B cell environments appears to act through an interaction with a p53 interacting protein; however we and others have shown that BL clones frequently carry mutations of p53 which would render any modulation of the p53 pathway redundant (Lindstrom and Wiman, 2002). Furthermore we have also previously demonstrated that the protection offered to EBV-positive clones by EBV shields cells only from p53-independent apoptosis. Our data suggests therefore that EBNA1 is not the agent responsible for apoptosis protection in BL cells.

(ii) EBERs

By using the same pRTS vector system, we were able to express variable levels of EBERs in EBV-loss clones from two BL cell backgrounds. Low level EBER expression was found in the absence of dox probably due to low level activation of the natural EBER promoters. We could however increase EBER expression to roughly twice that of an EBV-positive clone by titrating dox into the cell medium.

In previous studies, an expression vector carrying multiple copies of the EBERs was required to restore physiological EBER expression to EBV-loss clones (Kitagawa et al., 2000; Komano et al., 1999; Samanta et al., 2006; Samanta et al., 2008). Alternatively an EBER ori-P expression vector was transfected into cells already expressing EBNA1, a process requiring two rounds of drug selection (Ruf et al., 2000; Ruf et al., 2005). Using the pRTS expression system however, we can constitutively

express EBNA1 and conditionally express physiological levels of the EBERs with only a single copy of the EBER cassette and can generate stable cell lines without the need for drug selection. This provides a novel and arguably more robust method to study the effect of EBER expression on apoptosis resistance in BL cells.

One observation made during the validation of EBER expression was that the relative expression of EBER2 compared to EBER1 in pRTS EBER CD2 transfected cells was greater than was observed in naturally occurring EBV-positive clones of the same BL lines. This could be a result of either high EBER1 expression in naturally occurring EBV-positive clones or disproportionately high EBER2 expression from the pRTS EBER CD2 vector. However, despite this change in ratio, the vector is capable of generating quantities of both EBER1 and EBER2 greater than are observed in EBV-positive clones. The relative level of EBER1 and EBER is therefore unlikely to bias the investigation into the effect of EBERs on apoptosis resistance; indeed the change in ratio may well have gone un-noticed but for the highly accurate QRT-PCR assays used to measure EBER1 and EBER2 expression. To corroborate the results of the QRT-PCR analysis it may also be informative to carry out northern blot analysis of EBER expression in pRTS EBER CD2 transfected cells. This technique could also be used to ensure that the EBER transcripts generated by this vector are of the correct size and are therefore likely to be present in their active conformation.

Using pRTS EBER CD2 we found that expression of EBERs, even at levels roughly twice those observed in EBV-positive clones, was insufficient to protect cells from apoptosis induced by ionomycin, roscovitine or anti-IgM. This is contrary to several previous reports, in which EBERs were found to be the viral gene product at least partially responsible for EBV-mediated resistance to cycloheximide, glucocorticoid, hypoxic stress, serum deprivation and IFNα (Kitagawa et al., 2000; Komano et al., 1999; Nanbo et al., 2005; Ruf et al., 2005). Investigation into the proposed mechanism of EBER-mediated protection from apoptosis provides clues to the possible reasons for the discrepancy between our study and the bulk of the work present in the literature. EBERs are currently believed to induce apoptosis resistance through interaction with PKR and increased production of IL-10. The interaction of EBERs with PKR however remains controversial; EBERs clearly bind PKR in cell free systems (Sharp et al., 1993; Vuyisich et al., 2002), but EBERs have yet to be detected in association with PKR in latent infections. In addition, use of phosphorylation specific antibodies

reveals that EBERs are unable to prevent phosphorylation and thus activation of PKR during IFNα treatment (Ruf et al., 2005). In BL cells, it is also likely that most if not all EBER transcripts are bound to the ubiquitous ribosomal protein, L22 (Elia et al., 2004).

Binding of EBERs to L22 has been shown to attenuate their ability to associate with PKR (Elia et al., 2004). The induction of IL-10 production by EBERs was investigated in the final section of this chapter.

(iii) IL-10 and vIL-10

Previous reports have indicated that EBV is able to up-regulate expression of IL-10 in BL cells (Kitagawa et al., 2000). Up-regulation of IL-10 is believed to occur through the binding of the EBERs to RIG-I (Samanta et al., 2006; Samanta et al., 2008). We found however, that EBV status or EBER expression had little or no effect on expression of IL-10 or IL-10R. The inability of EBERs to modulate expression of IL-10 could explain the inability of EBERs to restore resistance to apoptosis to EBV-loss clones in our experiments. Our findings further suggest that a viral gene product other than EBNA1 or the EBERs must be responsible for apoptosis resistance in our EBV-positive BL clones.

During this analysis of IL-10 we also investigated expression of vIL-10, which has nearly 90% colinear aa sequence identity to its cellular counterpart (Figure 5.23). vIL-10 is also an autocrine growth factor capable of increasing resistance to apoptosis in the same manner as cellular IL-10, but is believed to be expressed only during the EBV lytic cycle (Ding et al., 2000; Ding et al., 2001; Stuart et al., 1995).

We found no correlation between detection of the viral and cellular IL-10 transcripts, indicating there is no crossover between these two RT-QPCR assays; however vIL-10 expression levels were higher than we would have expected from the low level lytic activity observed in EBV-positive clones in chapter 1. It is possible therefore that apoptosis resistance in EBV-positive clones could be mediated by low level expression of the EBV lytic cycle proteins. Using QRT-PCR we found that expression of most lytic cycle genes was low, probably due to the low expression of the major activator of lytic replication, BZLF1. Interestingly however, we found significant expression of all three of the late lytic cycle antigens we investigated (vIL-10, BVRF2 and gp350). This indicates that EBV-positive BL clones contain cells transiting the whole lytic cycle and implies that the observed expression of vIL-10 is likely to be part of an ongoing low level lytic activity within EBV-positive BL clones. By comparison to lytic

LCL standards with 2% of cells in lytic cycle we can infer that only a very small percentage of cells are likely to be actively participating in this lytic cycle activity. However, like its cellular counterpart, vIL-10 is secreted into the cell medium and could affect surrounding cells through paracrine signalling mechanisms; hence it could still provide a mechanism for EBV-mediated apoptosis resistance in EBV-positive cells.

We found that inhibition of the entry into late lytic cycle using ACV, or exposure of EBV-loss cells to supernatant medium (presumably containing vIL10) from EBV-positive clones, was unable to restore resistance to apoptosis. This suggests that vIL-10 is unlikely to be responsible for resistance to apoptosis in EBV-positive clones. Indeed, in subsequent work in our laboratory, a dox-regulated vector expressing vIL10 has been stably introduced into EBV-loss Akata-BL cells and, following induction of vIL10 synthesis, has not offered the cells any apoptosis resistance (G.Kelly, personal communication).

These uniformly negative results cannot be dismissed by the argument that an approach based on the dox-regulated vector system is somehow incapable of revealing anti-apoptotic functions. In fact, this very approach has clearly mapped the protection mediated by Wp-restricted Latency (as seen in a minority of eBL tumours) to Wp-driven expression of BHRF1 (Kelly et al., 2009 (in press)), the EBV-coded bcl2 homologue protein normally associated with the virus lytic cycle. We therefore feel that the negative results described here are significant negatives, and that the protection being offered to BL cells by Latency I EBV infection cannot be attributed either to EBNA1 alone, to EBNA1 in combination with the EBERs, to viral IL10 expression, or to virus-induced cellular IL10 expression.

Final conclusions

Factors which contribute to the pathogenesis of BL

Denis Burkitt first described eBL tumours in the jaws of African children and mapped its incidence to areas holoendemic for malarial infection (Burkitt, 1958; Burkitt, 1962b; Burkitt, 1962a). Analysis of this prevalent malignancy revealed the presence of EBV within virtually every tumour (Epstein et al., 1964b; Magrath, 1990; Raab-Traub and Flynn, 1986). Subsequently two further forms of BL were discovered (sporadic BL and AIDS-associated BL), which were associated with EBV to a lesser extent. The less common sporadic worldwide form of the disease has an association with EBV which varies with geographical location from 15-20% in the West to 85% in some areas of South America. It has been proposed that, in these higher incidence areas, other parasitic infections may again promote the EBV-associated form of the disease. AIDS-associated BL is unusually prevalent in those infected with human immunodeficiency virus (HIV) and has an association with EBV of 30-40%. Common to all three forms of BL is deregulated c-Myc expression resulting from a reciprocal translocation that places the c-myc gene under the control of the Ig loci. Thus BL pathogenesis appears to be a product of genetic changes, viral infection and environmental factors.

(a) Role of non-viral factors in BL pathogenesis

Central to the pathogenesis of BL is the c-myc translocation; however its origin is not fully understood. The detection of c-myc translocations in tonsillar B cells suggests that they may be a common aberrant by-product of normal B cell development (Goossens et al., 1998). These translocations were confined to GC B cells implying that translocation occurs during GC transit as a consequence of erroneous class switching or somatic hypermutation (SHM). In agreement with this observation, c-myc breakpoints in sporadic and AIDS-associated BLs commonly occur within the first exon or intron of the c-myc gene and within class switch regions of the Ig_H locus (Joos et al., 1992; Magrath, 1990; Pelicci et al., 1986; Shiramizu et al., 1991). Interestingly however, eBL breakpoints generally occur more than 100kb upstream of the first exon of the c-myc gene and in the VDJ region of the Ig_H locus, implying that translocation may occur in the bone marrow during VDJ recombination. Despite the differing

breakpoints however, all BL cells strongly resemble cells of GC origin; they possess rearranged hypermutated Ig genes, often with indications of continuing Ig gene diversification (Klein et al., 1995; Tamaru et al., 1995). In addition all forms of BL display a GC pattern of cellular gene expression when analysed by microarray (Kuppers, 2003; Kuppers, 2005). A GC origin for BLs could also explain the role of holoendemic malaria and HIV infection as risk factors for BL development. Holoendemic malaria is known to act as a chronic stimulus to the B-cell system and, by analogy to other parasitic infections (Araujo et al., 1999), is likely to increase GC activity. In addition, malaria may also suppress EBV-specific T cell responses, although this remains controversial (Geser et al., 1989; Gunapala et al., 1990; Moormann et al., 2007). In a similar manner, infection with HIV also acts as a chronic stimulus to the B-cell system. Long before any obvious T-cell impairment, HIV patients frequently show expansion of GC regions (Ioachim et al., 1990) and an increase in EBV load within latently infected B cells (Piriou et al., 2004).

In BL cells c-myc is believed to influence the expression of nearly 15% of cellular genes (Li et al., 2003). Among these are large numbers of genes involved in growth and proliferation which are responsible for the high proliferative index observed in BL cells. These changes include an up-regulation of the translation initiation factors eIF-4E and eIF-2α, which are important in cell growth (Rosenwald et al., 1993; Jones et al., 1996) and cyclin D2 and CDK2, which are essential for cell-cycle progression (Steiner et al., 1995; Berns et al., 1997) and a repression of the cyclin-dependent kinase inhibitor (CKI), p21, (Wu et al., 2003). Overexpression of c-Myc, however, also activates several apoptotic checkpoints, which in normal cells would protect against aberrant c-myc expression. High level myc expression activates the p53 pathway through the nucleolar tumour suppressor, ARF (Eischen et al., 1999). In addition c-Myc down-regulates expression of the anti-apoptotic Bcl-2 family member proteins, Bcl-2 and Mcl-1 (Eischen et al., 2001; Maclean et al., 2003) and up-regulates expression of the pro-apoptotic Bim and Bax proteins (Juin et al., 2002; Mitchell et al., 2000). To counter these apoptotic signals, BL cells frequently carry a mutation of c-myc which prevents the up-regulation of the Bim protein (Adhikary and Eilers, 2005; Hemann et al., 2005). Those that have a WT c-myc often carry a mutated and non-functional p53 or ARF gene or overexpress the p53 degradation targeting protein, MDM2 (Eischen et al., 1999; Lindstrom et al., 2001). However despite these genetic changes BL cells remain highly sensitive to apoptosis both *in vivo* and *in vitro*.

(b) The role of EBV in BL pathogenesis

The precise role of EBV in BL remains enigmatic; however the high frequency of EBV association with BL (particularly the endemic form) strongly implies a role for the virus in tumour pathogenesis. Indeed, eBL patients have unusually high antibody titers against the EBV capsid antigen (VCA) and children who developed BL had significantly raised anti-VCA titers months or years before the clinical onset of the tumour (de The et al., 1978; Geser et al., 1982). In addition, EBV-positive tumours carry monoclonal EBV genomes within every cell (Magrath, 1990; Raab-Traub and Flynn, 1986). However, unlike EBV transformed B lymphocytes and PTLD, where proliferation is driven by the EBV growth transforming proteins, proliferation in BL cells is driven by c-Myc. Most BL cells also lack expression of nearly all latent antigens, including the major EBV growth transforming proteins, EBNA2 and LMP1. These BLs express a highly restricted form of viral gene expression (termed Latency I), only expressing the EBNA1 protein from the Q promoter along with the non coding EBERs, the BARTs and a selection of virally encoded miRs. A second subset of BL tumours show an alternative form of viral transcription (termed Wp restricted latency). They express EBNA1, the EBNA3 proteins, a truncated EBNA-LP, BHRF1, the EBERs, BARTs and miRs (Kelly et al., 2002). Expression of the viral gene products in Latency I or Wp restricted latency by themselves appears insufficient to induce tumourigenesis in BL cells; however the ubiquitous presence of EBV within eBL tumours indicates that this association is not coincidental.

Several scenarios have been proposed to explain the contribution of EBV to eBL. A popular scenario is that EBV initially establishes a Latency III infection in resting B cells, leading to an expansion of the infected B cell pool. Chronic antigen stimulation and immune suppression through agents such as malaria may magnify this effect. Indeed EBV viral load in healthy carriers in holoendemic areas is tenfold higher than in HLA matched UK controls (Njie et al., 2009) and EBV load rises to IM like levels in acute malaria patients suggesting that between 1-10% of B cells may be harbouring EBV (Hochberg et al., 2004). Parasitic antigen stimulation may then induce B cells to transit a GC where the c-myc gene and Ig enhancers are juxtaposed, translocated and mutated during aberrant class switching and/or SHM. As shown by the forced expression of c-myc in LCLs (Polack et al., 1996; Schuhmacher et al., 1999; Pajic et al., 2001), Latency III infection is incompatible with c-myc expression. This

incompatibility with c-myc, coupled with the high immunogenicity of several of the EBV latent antigens could lead to the selection of rare cells which have down-regulated viral gene expression either to a latency I infection or to Wp restricted Latency through deletion of EBNA2 (Kelly et al., 2002; Kelly et al., 2005; Kelly et al., 2006). Additional changes such as mutation of the p53/ARF pathway may then allow the outgrowth of latently infected cells.

An alternative scenario is one where the c-myc translocation occurs in EBV infected cells outside the GC. In this scenario, the GC phenotype is imposed by the presence of high c-Myc expression, a phenomenon which has previously been demonstrated in B cells *in vitro* (Polack et al., 1996; Schuhmacher et al., 1999; Pajic et al., 2001; Klein et al., 2003).

A third possibility is that the c-myc translocation (possibly encouraged by the presence of a chronic parasitic stimulation or HIV infection) occurs prior to EBV infection. Different EBV isolates have been observed in a case of Burkitt-type acute lymphoblastic leukemia (Roncella et al., 1993), while the Oma-BL tumour was found to contain both EBV-positive and EBV-negative cells (Trivedi et al., 1995), suggesting that EBV infection may have occurred late in tumour development. EBV infection in a c-myc translocated cell could drive cells into full malignancy either through direct Latency I infection or via a transient Latency III infection which is subsequently down-regulated to Latency I.

(i) EBV-loss from BL cell lines and its implications for BL pathogenesis

Whatever the role of EBV in BL pathogenesis, it is clear that EBV infection occurs prior to full cellular transformation as virtually all EBV-positive tumours carry monoclonal EBV genomes within every cell (Magrath, 1990; Raab-Traub and Flynn, 1986). However, the fact that EBV is not lost from BL cells *in vivo* also suggests that it contributes to the continued growth and survival of BL cells. Indeed most EBV-positive BL cell lines, when cultured *in vitro*, retain EBV and are unable to tolerate its loss (Nasimuzzaman et al., 2005). Interestingly however, cases of EBV-loss have been observed in rare BL cell lines *in vitro* and provide an interesting opportunity to study the continued role of EBV in BL growth. First reported in a late passage of the sporadic Akata-BL cell line (Shimizu et al., 1994), loss of EBV has also been observed in endemic cell lines (Kitagawa et al., 2000) and from cells in early passage (Kelly et al., 2006).

In this study, we investigated spontaneous EBV-loss from a panel of 12 EBV-positive, Latency I BL cell lines from early, mid and late passage, which represents a much greater diversity of BL cell lines than previous studies. In most BL cell lines, (especially those in early passage) we did not observe EBV-loss suggesting that in these cases EBV is still absolutely required for continued cell growth *in vitro*. It also suggests that *in vivo*, EBV is actively participating in continued BL pathogenesis and not merely a passenger left over from the initial expansion of the B cell pool by Latency III infection.

We noticed that the propensity of cells to lose EBV appeared to be dependent on the time that cells had spent in culture. This indicates that mutations acquired *in vitro* may possibly fulfil roles that were essential for BL cell survival during BL pathogenesis and early passage growth in culture. We also observed that clones from cell lines which yielded EBV-loss clones had a greater variability in their viral load indicating that ability to lose EBV was influenced by their ability to efficiently segregate equal numbers of EBV genomes to daughter cells upon cell division. Thus, the potential for a cell line to lose EBV may be governed by its requirement for EBV for continued cell growth. However, the probability that a cell which could potentially survive without EBV will lose the virus may be a product of how uniformly EBV genomes are segregated during cell division.

We may in future be able to force EBV-loss from cell lines that in this study failed to yield EBV-loss clones using dominant negative EBNA1 or anti-EBNA1 siRNAs. Comparing EBV-positive and EBV-loss clones generated using these methods may allow us to better understand the role of EBV *in vivo* before the onset of cellular changes acquired in culture.

(ii) Phenotypic effect of EBV-loss in BL cells

Loss of EBV in Akata-BL is reported to increase susceptibility to apoptosis and decrease tumourigenicity in SCID mice and survival ability in low serum and soft agar (Komano et al., 1998; Shimizu et al., 1994). EBV-loss in endemic cell lines also has similar effects (Kelly et al., 2006; Kitagawa et al., 2000), implying that expression of the limited number of viral transcripts in Latency I is capable of enhancing the growth transforming potential of BL cells. These observations are compatible with any of the models of EBV-mediated BL pathogenesis; however the observation that Latency I EBV infection enhances tumourigenic potential indicates that EBV may drive the final stages of the malignant conversion of BL cells.

Comparisons of EBV-positive and EBV-loss clones suggest that the increased tumourigenicity of EBV-positive cells results from increased expression of the anti-apoptotic Bcl-2 protein (which is deregulated in a number of malignancies) and through a cell cycle-dependent modulation of c-Myc expression (Ruf et al., 1999). A more recent study also implicated the proto-oncogene, Tcl-1 (Kiss et al., 2003); however no mechanism for regulation of these genes by EBV has yet been suggested.

In this study, we observed that in most cell backgrounds EBV-positive and EBV-loss clones have very similar growth and survival properties. Contrary to the above reports, this indicates that the presence of Latency I transcription is unlikely to be sufficient to drive cells into a fully transformed state. The consistent increase in apoptosis sensitivity in EBV-loss clones suggests instead that the primary role of EBV in BL pathogenesis is anti-apoptotic.

In BL cells the primary drive for cells to enter apoptosis is through the overexpression of c-myc. Myc induces apoptosis primarily through the p53 and/or Bim/Bax pathway; however we did not find changes in expression of these proteins upon EBV-loss. It is possible that EBV is able to provide a measure of protection against p53 or Bim/Bax mediated apoptosis until such time as cellular mutations fulfil that role. This hypothesis could also explain the absence of EBV from a large percentage of sporadic BL tumours. However a hit and run hypothesis for EBV pathogenesis cannot explain the fact that EBV continues to provide protection from apoptosis when cell lines are cultured *in vitro*. This indicates therefore, that the apoptosis protection afforded to BL cells by EBV must act through a mechanism which is independent of the traditionally recognised machinery of c-myc induced apoptosis. This is supported by the observation that EBV appears unable to protect cells from p53-mediated apoptosis pathways.

In the future we would like to characterise in more detail the apparent ability of EBV to protect cells from apoptosis induced through p53-independent mechanisms, but not from cytotoxic drugs which induce p53-mediated apoptosis. In light of the observation that all the BL clones investigated have some disruption of the p53 pathway, it would initially be informative to investigate whether BL cells can functionally express the p53 homologues, p63 and p73. These investigations could provide interesting information on disruption of c-Myc induced apoptosis during BL pathogenesis and EBV's possible role therein. In addition the mechanisms of apoptosis in BL cells could be further investigated using

alternative apoptosis inducing agents, specific inhibitors of apoptosis (such as caspase inhibitors) or by monitoring key events in apoptosis progression, such as calcium efflux or cytochrome c release.

(d) Mechanism of Apoptosis protection in EBV-positive clones

Despite the high level of interest in the effect of EBV in BL pathogenesis, there are currently no published reports which have investigated cellular gene expression between EBV-positive and EBV-loss clones, by gene expression profiling. Using the Affymetrix system we analysed the cellular gene expression in paired EBV-positive and EBV-loss clones from multiple BL backgrounds. By comparison to a published report of gene expression in a large number of BL tumours, we found that all BL samples regardless of EBV status, tumour origin or time spent in culture displayed a typical BL pattern of gene expression. In agreement with previous studies (Polack et al., 1996; Schuhmacher et al., 1999; Pajic et al., 2001), this data suggests that the BL phenotype is imposed by deregulated c-myc expression.

The surprising observation that there were no consistent differences in cellular gene expression between our EBV-positive and EBV-loss clones suggests that differences in apoptosis resistance between EBV-positive and EBV-loss clones may not be caused by transcriptional regulation of cellular genes by EBV. It is possible that differences in gene expression may only be visible during induction of apoptosis. To investigate this possibility it may be feasible to investigate protein levels of key apoptotic genes, such as Bcl-2 family member proteins after an apoptotic stimulus. It is also possible that apoptosis resistance may be caused by some post-translational mechanism. This could be through a direct protein-protein interaction by EBNA1 or possibly a BART encoded protein, an RNA-protein interaction by the EBERs, an inhibition of mRNA processing by the EBV-encoded microRNAs or through a yet to be fully characterised mechanism, for example, low level expression of a lytic cycle antigen, such as vIL-10. If this were the case, it is possible that these changes could be revealed using a proteomic approach, such as 2D gel analysis or mass spectrometry.

Several studies have shown that apoptosis protection in EBV-positive clones is in fact mediated by EBV by demonstrating that reinfection of EBV-loss clones with recombinant EBV restored Latency I infection and apoptosis resistance (Kitagawa et al., 2000; Komano et al., 1998; Ruf et al., 1999). Contrary to these reports, we found that reinfection with EBV *in vitro* results almost exclusively in

Latency III viral gene expression. This observation supports a model of pathogenesis in which EBV infection occurs before the c-myc translocation as no BL tumours have been observed with a Latency III pattern of viral gene expression.

By investigating viral gene expression in large numbers of reinfections, we were able to isolate a rare Latency I infection event and found that it restored apoptosis protection to EBV-loss cells. When viewed in the context of the other published reports on reinfection of EBV-loss clones, this result demonstrates that the apoptosis resistance of EBV-positive BL clones is virally mediated. It would be informative to have more Latency I infected clones and infected clones from several BL backgrounds. The probability of producing a Latency I infection may be increased with a non-transforming recombinant EBV, such as a dual Cp Wp KO virus.

The only viral gene products whose role in tumourigenicity and apoptosis resistance has been investigated in the context of EBV-positive and EBV-loss clones are the EBNA1 protein and the EBERs. There is some evidence to suggest that EBNA1 may protect some cell lines from p53 mediated apoptosis (Kennedy et al., 2003; Saridakis et al., 2005); however it was not shown to have any effect on apoptosis in an EBV-loss background (Komano et al., 1998; Ruf et al., 1999). Currently most of the published data suggests a tumourigenic and anti-apoptotic role for the EBERs (Kitagawa et al., 2000; Komano et al., 1999; Nanbo et al., 2002; Ruf et al., 2005). They are believed to act through an up-regulation of IL-10 (Kitagawa et al., 2000; Samanta et al., 2008) and an interaction with the anti-viral PKR protein (Nanbo et al., 2002). These activities however remain controversial as EBERs expressed in the nucleus are largely bound to the ribosomal protein, L22 (Elia et al., 2004; Schwemmle et al., 1992). Nevertheless the current lack of published data on the anti-apoptotic effect of the BARTS and EBV-encoded miRs means that EBERs are still the prime candidates for EBV-mediated tumourigenicity and apoptosis resistance.

The surprising observation in this study, that apoptosis resistance could not be restored through expression of EBNA1 or EBERs, suggests that these Latency I gene products are not responsible for apoptosis resistance in BL cell lines. In addition it also seems that low level expression of lytic cycle proteins such as IL-10 is not responsible for apoptosis resistance, indicating a role for either the BARTs or the EBV encoded miRs. Of these two possibilities, expression of the miRs appears to be the most likely to elicit the anti-apoptotic effect observed in EBV-positive clones. The rapid escalation of

interest in the miRs has already led to the discovery of several potentially interesting miR functions (Choy et al., 2008; Xia et al., 2008) and new EBV-encoded miRs continue to be discovered, most of which have yet to be fully characterised. Obviously, the first step in determining the role of miR expression in apoptosis resistance is to characterise their expression in EBV-positive BL clones, which is becoming increasingly straightforward as detection technology improves.

Overall we have shown that EBV consistently provides protection from apoptosis in BL cells. This phenotype could not be linked to changes in cellular gene expression and could not be reconstituted by expression of EBV latent gene products previously associated with apoptosis resistance. Thus, it becomes increasingly likely that EBV-mediated apoptosis resistance in BL cells is regulated by a novel mechanism.

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